Breast Cancer:

A Multidisciplinary Approach

Edited by

G. St-Arneault P. Band L. Israel

With 74 Figures



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Chapter 1
The Magnitude of the Breast Cancer Problem

S. J CUTLER S. S. DEVESA and T H. C. BARCLAY

The medical profession and the general public are justly concerned with the control of cancer of the female breast The sheer magnitude of the problem demands that it be given a great deal of attention We estimate that in the United States 665 000 new cancers will be diagnosed in 1975 of which 88 700 or more than 1 out of every 8 will be a cancer of the female breast Among women breast cancer accounts for more than 1 out of every 4 cancers

These figures are based on the Third National Cancer Survey which provides information on the incidence of cancer during the 3-year period 1969-1971 in nine areas in different parts of the United States with a combined population of 21 million people By relating the number of breast cancers to the female population in the surveyed areas we obtain an annual incidence rate of 73 8 per 100 000 female population[1], or 738 per million[1]

The incidence rate varies markedly with age The risk of breast cancer is low in young women and increases continuously during the life span We see in Figure 1 that the annual incidence rate is less than 50 per 100 000 women at age 35 approaches 200 per 100 000 by age 55 and gets close to 300 per 100 000 by age 75 Cumulating the risk of occurrence of breast cancer through a woman s life span and taking into account the life expectancy of women in the United States we find that the lifetime probability of developing cancer of the breast is 7 2 % i e an American woman has 1 chance in 14 of developing breast cancer during her lifetime

The incidence pattern portrayed in Figure 1 reflects experience around calendar year 1970 What was the situation in earlier years? The first estimates of cancer incidence in the United States were obtained in a survey of 10 metropolitan areas during the calendar years 1937-1939 (DORN and CUTLER 1959) The same 10 areas were surveyed in 1947-1948[2] (DORN and CUTLER 1959) The third survey was carried out after an interval of more than 20 years in 1969-1971 (CUTLER et al 1975) In Figure 2 comparison is made of the age incidence curves for breast cancer in white women based on the data from the three surveys We see that between ages 40 and 55, a continuing upward shift occurred Little change in breast cancer incidence occurred among women between ages 55 and 70 but at older ages the rates changed erratically - a very large increase from 1937 to 1947 followed by a decrease to an intermediate position We do not know whether the wide fluctuation in rates at the older ages is real or whether it reflects at least in part variation in completeness of case reporting and variation in the accuracy of the population census

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[1] Age-adjusted to the 1970 U S population

[2] Information was collected in each area for 1 year only

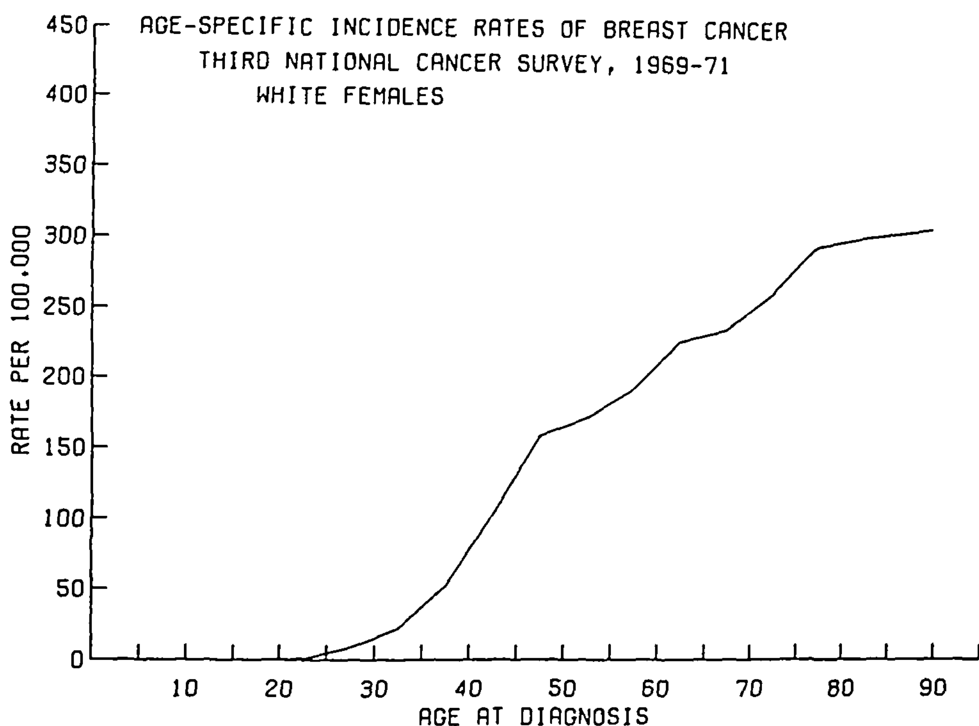


*Fig. 1 Age-specific incidence of breast cancer among whit
1969-1971 Third National Cancer Survey*

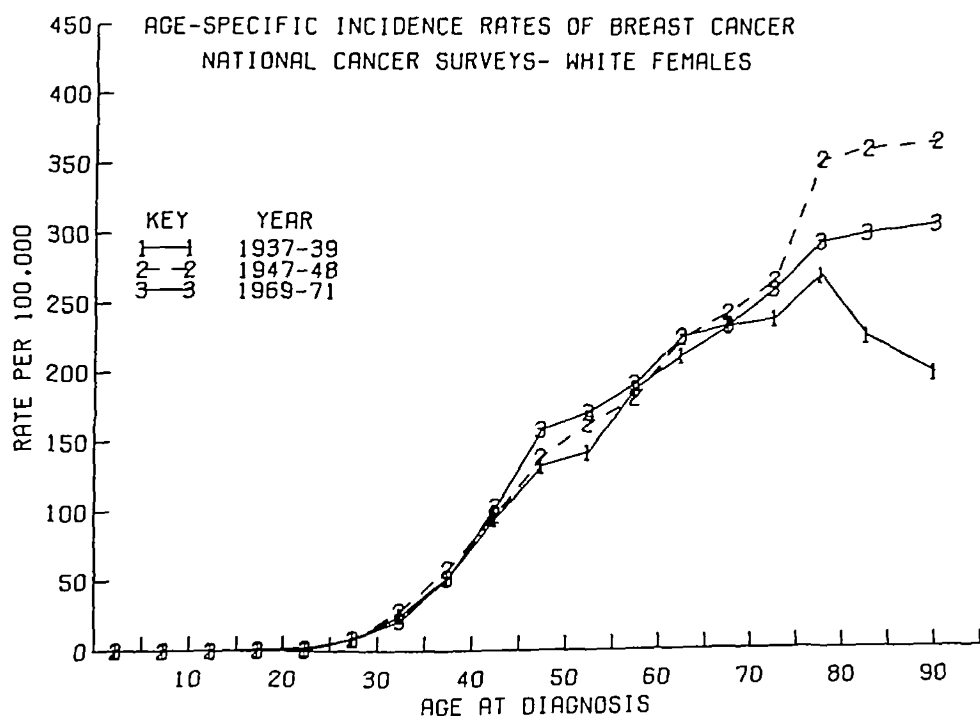


*Fig 2 Age-specific incidence of breast cancer among white
over time National Cancer Surveys*

Whereas the data from the three surveys are discontinuous, dence has been reported in the State of Connecticut for eac ginning with 1935. (See Publications of the Connecticut Sta ment of Health, Hartford, Connecticut in References) The p

of Connecticut was 3 million in 1970 Age-incidence curves for breast cancer are shown in Figure 3 for successive 5-year calendar periods 1935-1939 through 1965-1969 plus the 3-year period 1970-1972 There are eight curves on this chart and they do cross one another Nevertheless it is evident that breast cancer incidence has been increasing rather steadily between ages 45 and 65 At the older ages there has been considerable interweaving of the curves but the more recent data suggest that an upward shift may be developing

Similar data on the trend of breast cancer incidence is available for the Canadian province of Saskatchewan beginning with 1946 (Fig 4) In general the incidence curves have been moving up fairly steadily; and in contrast to the experience in Connecticut the rates at the older ages have increased substantially

Another way to look at the trend of breast cancer incidence at various ages is in terms of birth cohorts i e the rates experienced by women born in successive calendar periods This can be done by rearranging the data presented in the preceding charts For example the incidence rate at ages 50-54 in calendar period 1950-1954 pertains to women born around 1900; the rate at ages 60-64 in 1960-1964 also pertains to women born around 1900 In Figure 5 the Connecticut data are portrayed for birth cohorts around 1870, 1880 1890 1900 1910 1920 and 1930 The available data suggest that incidence of cancer for women born around 1870 1880 and 1890 was relatively stable - at least from age 65 on The data also indicate a clear and continuing upward shift of rates beginning with women born around 1900

The data for Saskatchewan are shown by birth cohorts in Figure 6 In contrast to Connecticut the upward shift is continuous and very marked over virtually all the ages and cohorts for which data are available

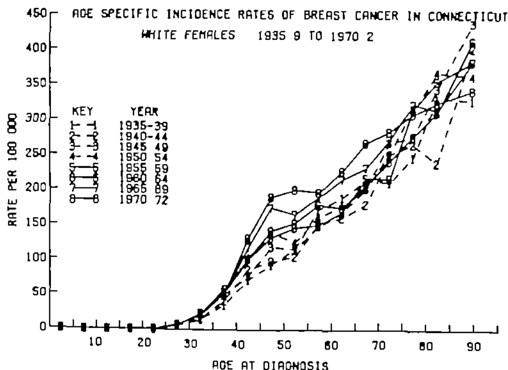


Fig 3 Age-specific incidence of breast cancer among white females in Connecticut 1935-1939 - 1970-1972

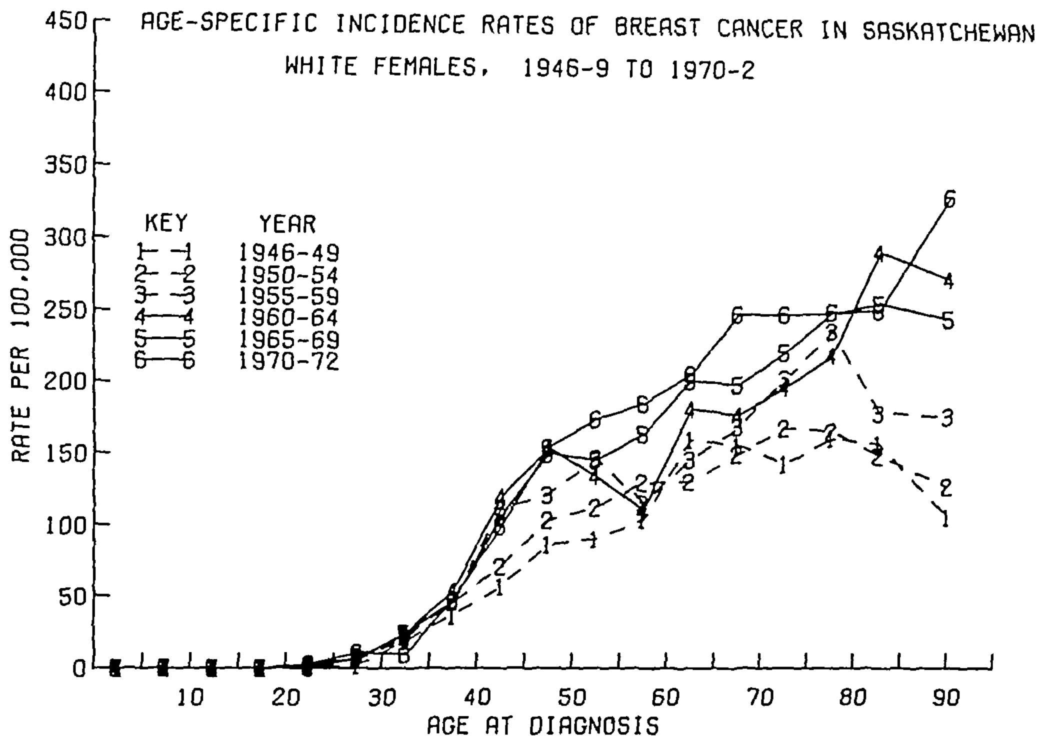


Fig 4. Age-specific incidence of breast cancer among white females in Saskatchewan, 1946-1949 - 1970-1972

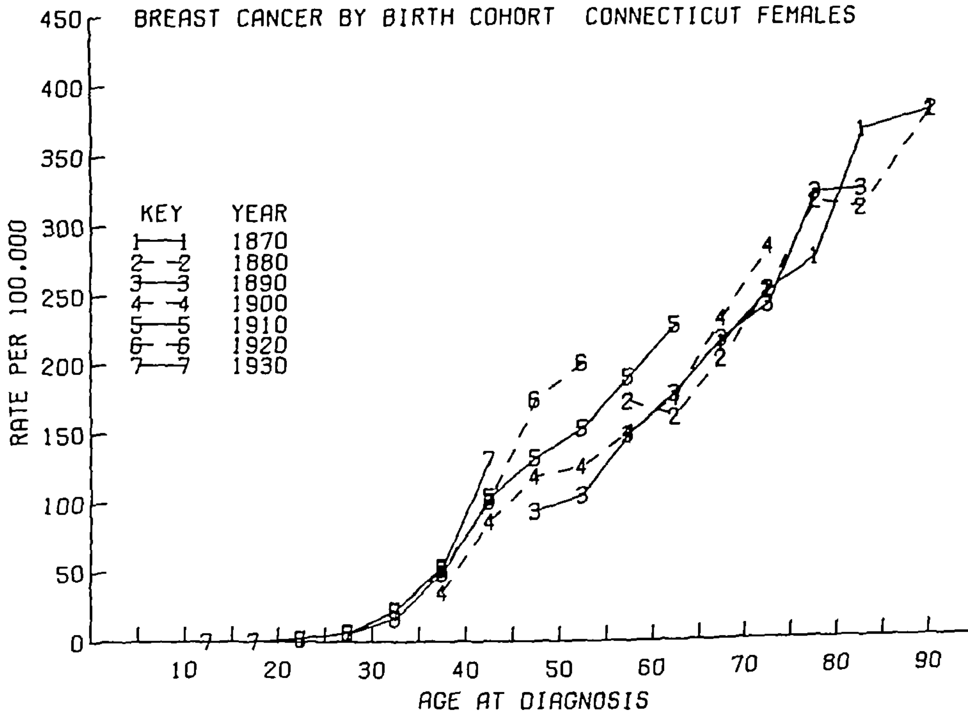


Fig 5 Age-specific incidence of breast cancer in Connecticut among birth cohorts, 1870-1930

In comparing trends in cancer incidence among several geographic areas, it is useful to consider the absolute values. The question is· are these

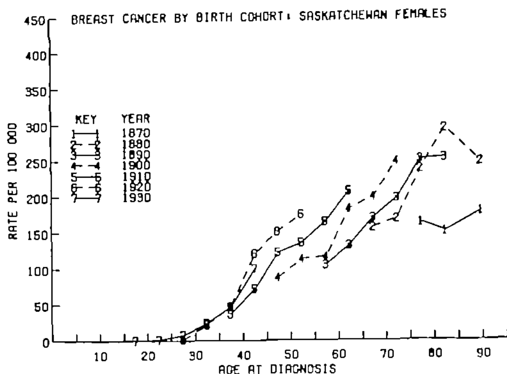


Fig 6 Age-specific incidence of breast cancer in Saskatchewan among birth cohorts 1870-1930

trends occurring at similar or at different levels of disease frequency? To facilitate comparison the age-specific rates have been consolidated to a rate for all ages combined Since the age distributions of the populations in the areas differ all the rates were adjusted to one standard age distribution - that of the United States in 1950 We see in Table 1 that the upward movement of breast cancer incidence in Saskatchewan began from a relatively low level of 42 4 per 100 000 in 1946-1949 The earliest rate from the U S surveys the rate for 1937 was much higher - 66 2 per 100 000 Thus the survey data point to a moderate increase from an initially high level and an apparent leveling

Table 1 Incidence of breast cancer over time in Connecticut Saskatchewan and National Cancer Survey

Years	Connecticut	Saskatchewan	National Cancer Surveys
1935-39	56 3	NA[a]	66 2 (1937)
1940-44	55 3	NA[a]	
1945-49	59 7	42 4[b]	72 6 (1947)
1950-54	64 7	47 5	
1955-59	64 0	56 3	
1960-64	66 4	61 0	
1965-69	74 0	64 5	
1970-72	79 6	69 1	72 0 (1969-71)

Note All incidence rates are per 100 000 (white) females age-adjusted to the 1950 U S population

[a] Not available

[b] 1946-1949

off at around 72 per 100,000. Although the rates increased rapidly in Saskatchewan, the latest incidence rate, for 1970-1972, is still slightly lower than the rate from the latest survey (69.1 vs. 72.0). In Connecticut, the rates were at an intermediate level during the 1940s, but are now somewhat higher than the rate from the latest survey (79.6 vs. 72.0).

The different trends observed in Connecticut, Saskatchewan, and in the national surveys suggest that populations living in different geographic areas have in fact had different experiences with respect to the level of breast cancer frequency and changes in the rate of occurrence over time. To explore this further, the rates in the individual geographic areas included in the three surveys were examined. Seven of the 10 areas in the first two surveys were also included in the third The changes in the reported rate of breast cancer incidence in each of these areas are shown in Figure 7 and compared with the trends in Connecticut and Saskatchewan. Two general patterns can be seen. The first is characterized by a continuing increase, as in Saskatchewan and Detroit. The second is characterized by a sharp increase, followed by a decrease to an intermediate level. Overall, there appears to be a tendency towards less diversity. The range of rates is now narrower than in earlier years; the rates appear to be converging towards a level of 70-75 breast cancers diagnosed per 100,000 women per year. One interpretation of this trend towards stabilization of the incidence rate is that women have been getting more homogeneous with respect to risk factors such as age at first pregnancy and diet, or that women with different risk factors are becoming more evenly distributed throughout the country as a result of population mobility.

Information on mortality from cancer of the breast is available over an extended number of years for each of the United States and for each

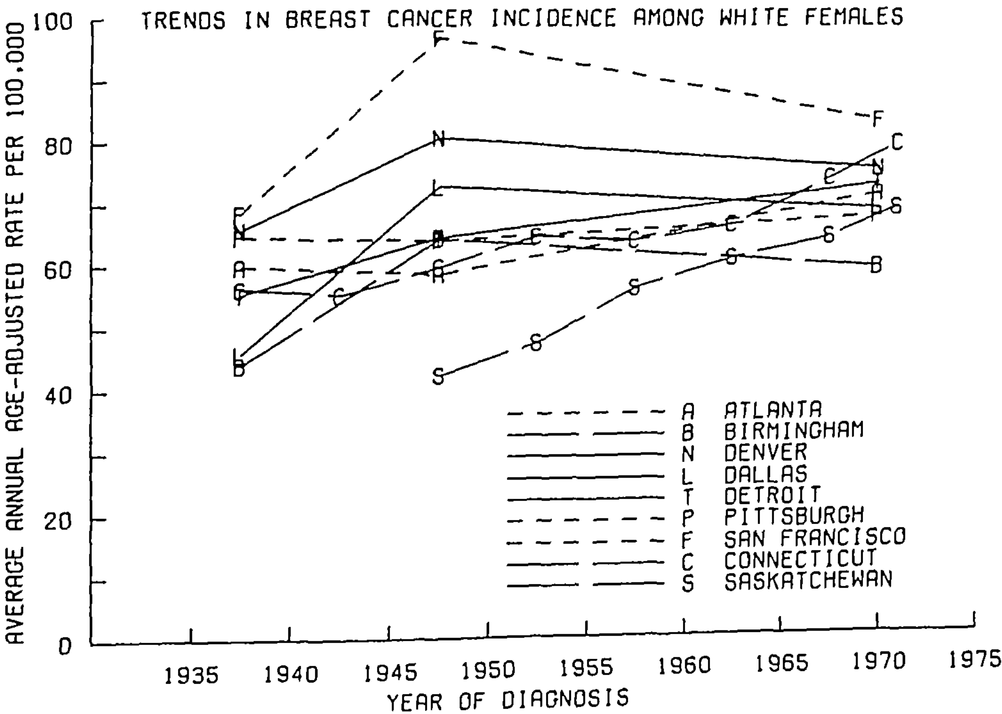


Fig 7 Trends in age-adjusted (1950 Standard) incidence rates of breast cancer in Connecticut, Saskatchewan, and the seven common areas of the National Cancer Survey

province of Canada Thus, information on changes over time is available for a wide range of geographic areas However mortality statistics are not a good indicator of changes in the rate of incidence of breast cancer as the ratio of reported incidence to registered mortality from breast cancer has been changing over time in those areas for which both types of data are available (Table 2) *This shift has been particularly*

Table 2 Trends in ratio of crude incidence to crude mortality rates for breast cancer in Connecticut and Saskatchewan Canada from CUTLER S J et al : Increasing incidence and decreasing mortality rates for breast cancer Cancer 28, 1378 (1971)

Time period	Saskatchewan			Connecticut		
	Crude incidence	Crude mortality	ratio[a]	Crude incidence	Crude mortality	ratio[a]
1946-1949[b]	37 0			66 1		
1951-1954		19 4	1 91		32 0	2 07
1950-1954	43 9			72 5		
1955-1959		24 2	1 81		31 2	2 32
1955-1959	51 8			71 8		
1960-1964		23 5	2 20		31 0	2 32
1960-1964	59 1			74 3		
1965-1969		25 0	2 36		31 5	2 36

[a] 5-year shift For example the incidence rate in 1950-1954 is related to the mortality rate in 1955-1959
[b] 4-year periods; all others are 5-year periods

Table 3 Trends in relative survival rates for female breast cancer 5-year relative survival rates[a] (No of cases)

	1940-1949	1950-1959	1960-1964	1965-1969
End Results Group	53% (12 184)	60% (22 105)	62% (13 326)	64% (14 493)
Connecticut	55% (4 926)	60% (7 516)	64% (4 896)	64% (5 915)
Saskatchewan[b]	50% (960)	59% (1 831)	62% (1 262)	63% (1 442)

1-year relative survival rate[a] (No of cases):

	1960-1964	1965-1969	1970-1971
End Results Group	88% (13 326)	89% (14 493)	90% (5 600)
Connecticut	90% (4 896)	90% (5 915)	89% (2 465)
Saskatchewan[b]	89% (1 262)	90% (1 442)	91% (629)

[a] Relative survival rates are observed survival rates which have been adjusted for the expected *normal* mortality of the group under study with respect to sex age calendar time and length of observation
[b] The survival rates for Saskatchewan are observed rates rather than relative i e they are not adjusted for *normal* mortality In addition the direct method rather than the life table method was used in their calculation

marked in Saskatchewan, where the ratio has increased from 1.91 to 2.36 in a period of 20 years. A similar increase in the ratio, though somewhat less dramatic, occurred in Connecticut. It is noteworthy that the ratios in these two areas are now the same - 2.36. The increase in the incidence/mortality ratio was due primarily to the increase in incidence, while the mortality rates have remained fairly stable[3].

The aforementioned divergent trends imply that fewer patients with cancer of the breast are now dying from the disease. Available data on patients followed via organized cancer registries indicate that this is true, i.e., patient survival rates adjusted for expected *normal* mortality have been increasing. In the United States, data on the survival of patients with cancer are available from a network of more than 100 hospitals of various types and sizes (End Results Group). Table 3 indicates that the 5-year survival rate increased from 53% for patients diagnosed in 1940-1949 to 60% for patients diagnosed in 1950-1959. Improvement since then has continued, but at a slower rate, reaching 64% among patients diagnosed 1965-1969. Data on the 1-year survival rate, available for more recently diagnosed cases (see Table 3), indicate that the improvement in patient survival appears to be continuing, but at a slow pace. The data from the hospitals in Connecticut, which contributed 38% of the breast cancer cases reported by the End Results Group, indicate a generally similar trend. Improvement in patient survival rates has also been reported in Saskatchewan (Table 3).

In an effort to ascertain whether the observed increase in incidence in Saskatchewan was at least partly attributable to changes in diagnostic criteria over time, BARCLAY et al. (1974) reported the review of tissue slides from benign and malignant tumours diagnosed during four time periods spanning 22 years, 1946-1968. Their findings indicated that the increase was not due to better case finding nor to changes in diagnostic criteria, but rather it appeared to be real.

In conclusion, while mortality from breast cancer is decreasing, the incidence of breast cancer is increasing in Saskatchewan, Connecticut, and several metropolitan areas of the United States Other areas of the USA have experienced relatively stable or even decreasing incidence rates. Whether these trends indicate a real convergence of rates or whether the observed trends will continue remains to be seen. Breast cancer remains, however, the most frequent site of cancer in women and deserves as much research attention as is possible.

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[3] In Saskatchewan, the mortality rate did increase from 1951-1954 to 1955-1959, but has changed little since.

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Chapter 2
Genetic Predisposition to Breast Cancer*

D E ANDERSON

The question of an inherited genetic basis for breast cancer has been debated for at least 50 years. Although familial aggregations of the disease have been described since the nineteenth century or longer, the debate seemingly commenced with the initiation of retrospective studies in which the frequency of breast cancer in the relatives of a large series of patients was compared with the frequency in some control group. These early studies indicated two- to fourfold risks for the disease among the relatives and that the increased risks applied only to breast cancer, i.e., the genetic effect was site-specific. (See reviews by CLEMMESEN, 1965; POST, 1966.) These studies were criticized on the grounds that the study and control groups were not comparable and/or the analytical procedures were inadequate. But later studies, such as those of WOOLF (1955), ANDERSON et al. (1958), TOKUHATA (1969), and MACKLIN (1959), using improved methodologies and more critical analytical techniques, yielded similar risks and again, evidence of a site-specific effect. The magnitude of these genetic risks was little different from those of other risk determinants including single vs married women, low vs. high parity, early vs. late menopause, late vs. an early age at delivery, or from comparisons of high vs. low socioeconomic class (MacMAHON et al., 1973). This lack of unequivocal evidence of a genetic effect, regardless of the type of study, may have helped to prolong the debate and nurture the notion that hereditary factors play only a minor role in breast cancer.

This notion is now being furthered by findings emanating from population comparisons of migrants to native-born women, where the breast cancer rates in migrants are approaching the rates of the locale or country to which they migrate (BUELL, 1973). This similarity is being interpreted as evidence of a strong environmental and a minimal genetic influence on breast cancer development (MacMAHON et al., 1973). However, population comparisons are no more useful for identifying a genetic component than they are for identifying the involvement of a virus or a specific dietary or other singular environmental factor. Population comparisons are influenced by many factors, including geography, culture, racial background and admixture, dietary factors, exposure to carcinogens peculiar to an area or life style, availability and use of medical services, types of services, methods of recording or reporting data, completeness of medical records, diagnostic and classification criteria, etc. Any factor being measured in such comparisons would be confounded with a host of other factors and would be difficult or impossible to identify specifically. At best, any detectable variation would have to be viewed on a multifactorial basis. One possibility not generally considered in such comparisons is that genetic factors could determine

* The original work cited in this report was supported in part by Grant GM 19513, C-1 and Contract NO1-CB-44004, from the National Institutes of Health.

the response of the host to specific geographical carcinogens absent in the original country but present in the new; for any genetically determined tumor the tumor itself is not inherited only the predisposition, and some further step(s) mediated through the environment are required for tumor development (KNUDSON et al 1973)

Population comparisons usually also pertain to neoplasms occurring at a given site (lung breast stomach etc) Certainly neoplasms at a given site may include a variety of histologic types each of which could be influenced by different causative factors (DOLL, 1972) Differences in tumor type and survival have been demonstrated between high- and low-risk populations (WYNDER et al 1963; MORRISON et al 1972) Consequently population comparisons because of the large environmental component and heterogeneity of tumor and clinical material are not meaningful for demonstrating or evaluating the importance of a genetic effect whatever the tumor site

Genetic evidence has to come from special investigations within populations where the environmental effects are not so diverse and are at least controllable to some extent Although an impressive number of special investigations have been conducted on breast cancer they too have suffered from the criticism of tumor and clinical heterogeneity Each study has been based on the implicit assumption that cancer of the breast whatever the clinical or morphologic type referred to a single homogeneous disease entity If the disease were indeed heterogeneous as data from various sources indicate (reviewed in ANDERSON 1975a) and not homogeneous as previously assumed then the effect of this erroneous assumption would be to dilute any measure of a genetic component This could be one reason for the failure of past studies to provide unequivocal evidence of a genetic effect in breast cancer development

GENETIC INVESTIGATIONS

Evidence is now beginning to accumulate indicating that when an attempt is made to reduce the heterogeneity among patients the risks may well exceed the oftcited two- to threefold levels (ANDERSON 1972 1974) One of the initial attempts in this regard involved classifying patients according to family history and clinical onset of the disease whether premenopausal or postmenopausal (ANDERSON 1971) The rationale for the latter classification was the suggestion by de WAARD et al (1964) that premenopausal breast cancer involved ovarian estrogens and postmenopausal disease estrogens of adrenal origin Patients with a family history of the disease were found to have an earlier age at onset than nonfamilial patients but of particular interest was the high rate of bilaterality among familial patients with premenopausal onset (15 5%) compared with familial patients with postmenopausal onset (4 8%) No association was observed between age and bilaterality in the nonfamilial group in which the bilaterality subsequently was found to exert a significant enhancing effect on the risks for breast cancer development in the relatives of patients (ANDERSON 1972) as summarized in Table 1

A range of risks is shown the smaller value pertaining to the verified breast cancer occurrences in first-degree relatives and the second value to both verified and unverified occurrences The percentages refer only to verified occurrences The percentages and risks are distinctly higher in relatives of patients with early and bilateral disease (9 5-fold) than in relatives of patients with unilateral and postmenopausal disease (1 3- to 1 9-fold) when both groups are compared with similar-aged

Table 1 Risks for breast cancer in first-degree relatives of patients with preme pausal and bilateral breast cancer and those with postmenopausal and unilateral breast cancer

Classification of patient	(C)ontrol relatives		(B)reast cancer relative		
	No at risk	Age adj % breast cancer	No at risk	Age adj % breast cancer	Risk B C
Premeno and bilateral	175	2 3	33	17 1	9 5[a]
Postmeno and unilateral	524	2.9	331	3 5	1 3 -

[a] p< 01
[b] p< 05

control relatives. If the two classes of patients are pooled, the re· sulting risks are reminiscent of those found in some previous studi i.e., approximately a two-fold risk, see review by POST (1966). Amo the familial patients, therefore, there is a subgroup with early an bilateral disease who manifest a strong genetic effect, but which i masked by the larger group in which the genetic effect is less pronounced.

That early and bilateral breast cancer should show a stronger genet effect than the unilateral type is in keeping with other neoplasms which a genetic basis has been convincingly demonstrated They are characterized by earlier onset and disease multiplicity, either in the form of bilaterality in a paired organ or multiple tumor foci i a single organ. Onset in patients with familial types of tumors may develop, on the average, anywhere from a few years to several decad earlier than the same tumor in unselected patients. These differing characteristics apply to childhood tumors as well (KNUDSON et al , 1973). Familial patients also manifest increased frequencies of multiple tumors. In familial melanoma patients, for example, 14% develc multiple primaries compared with about 2% in unselected patients. An patients with Sipple's syndrome (medullary thyroid carcinoma and phe chromocytoma), 75-80% exhibit multiple tumors vs. 12% in unselected patients; such differences also apply to other tumors, whether adult or childhood (ANDERSON, 1975b).

TWO-STEP MUTATION MODEL

KNUDSON (1971) advanced a model to explain the occurrence of heredit and nonhereditary tumors. His model proposes that all tumors origina from a single cell and are the consequence of two mutational events. The first may involve a germinal cell and thus be inherited, in whic case it would be present in every cell of the recipient. Or, the fir event may involve a somatic cell and all subsequent daughter cells, but it will not be hereditary. The second event in either case is so matic. Since one mutation already exists in all cells of patients wi heritable tumors, only a single second event is necessary for tumor development; consequently, heritable tumors will be early and freque multiple in occurrence The nonheritable type, however, requires two infrequent mutational events in a single cell for tumor development, and as such will be late and single in occurrence The model also pr dicts that carriers of the first mutation may develop no tumor, one, two or more in accordance with a Poisson distribution This two-step

model has been applied to three childhood tumors (KNUDSON 1971; KNUDSON and STRONG 1972a b) with excellent agreement between observed and expected numbers of gene carriers with zero one two or more tumors; and as expected gene carriers also had earlier onset and an excess of multiple tumors The proposal was made that perhaps all multiple tumor occurrences involve a germinal mutation The model could have applicability to heritable adult tumors because of their characteristic early and multiple occurrence

HEREDITARY TYPES OF BREAST CANCER

The relation of early age and multiplicity to genetic etiology is also shown by more recent studies (ANDERSON 1974) where in a further attempt to reduce heterogeneity patients were classified according to the pattern of breast cancer in their families i e who in the family had breast cancer prior to that in the patient Three basic familial patterns were utilized where prior disease involved (1) a second-degree relative of the patient (2) a sister of the patient and (3) the mother of the patient The question asked in each of these three pedigree groups was what is the risk for breast cancer development in the remaining sisters of the patients? The results of this analysis are summarized in Table 2

The controls in this analysis are the same as those previously described (ANDERSON 1974) and referred to in Tables 1 and 3 The high 40-fold risks in the 20-39 age class for the mother pedigree group pertain to sisters of patients who had premenopausal and bilateral breast cancer This is the only pedigree group in which these two characteristics had an enhancing effect on risk Clearly this pedigree group refers to a very high-risk group of women whose average risk is sixfold higher than that of controls If this sixfold risk is applied to the general population where the lifetime probability of breast cancer development to age 72 is about 6% then the lifetime probability to the sisters in the mother pedigree group would be on the order of 35% Not only is the likelihood of the disease high but the disease in this group also apparently develops relatively early in life Furthermore among the patients and their affected sisters the disease tends to develop at similar ages as evidenced by a correlation of $r = 0\ 45$ ($p < 01$) The disease in these affected relatives is also frequently bilateral 12-15%) and the affected mothers themselves manifest a higher than expected rate of bilaterality ($p < 05$)

In the sister pedigree group where the average risks are 2 3-fold higher than controls suggesting a 12% lifetime risk the correlation of age onset in the affected sisters was $r = 0\ 14$ not significantly different from zero and the bilaterality rate did not exceed expectation The disease in this group thus appears to be more variable unilateral in type and later-occurring than that in the mother pedigree group The results for the second-degree group are not meaningful because of the small number of affected individuals There is evidence of a definite age gradient in the results because the risks are higher at young than at old ages whereas in controls disease frequency increases with an age increase However Table 2 fails to account for the fact that women who were in the 40-59 or 60-79+ age classes at the time of their last observation also passed through the 20-39 age class without developing the disease; as such the table fails to provide a realistic appraisal of the age-specific risks Table 3 provides the age-specific probabilities and risks for the sister and the mother pedigree groups

Table 2 Risks of breast cancer in sisters of patients according to pedigree type and age of sister at risk at time of last observation

Age of sister at risk	Second-degree			Sister			Mother		
	No sisters at risk	No with breast cancer	Risk (vs controls)	No sisters at risk	No with breast cancer	Risk (vs controls)	No sisters at risk	No. with breast cancer	Risk (vs controls)
20-39	22	1	5 3	19	1	6 2	23	6	39.5[a]
40-59	80	1	1 4	70	4	2 3	96	15	7.0
60-79+	59	0	--	110	5	1.7	88	5	2 1
Total	161	2	0 5	199	10	2 3	207	26	6.1[a]

[a] p < 01.

Table 3 Age-specific probabilities and risks for breast cancer development according to pedigree type

Age class	Controls			Sister pedigrees				Mother pedigrees			
	No at risk	No with breast cancer	Prob breast cancer during age interval a	No at risk	No with breast cancer	Prob breast cancer during age interval b	Risk b a	No at risk	No with breast cancer	Prob breast cancer during age interval c	Risk c:a
20-29	338	0	000	195	1	005	—	206	0	000	—
30-39	312	0	000	186	0	000	(1 7)[a]	194	6	031	(9 9)[b c]
40-49	267	1	004	166	3	018	4 9	162	9	056	15 6[c]
50-59	190	3	016	132	1	008	0 5	114	6	053	3 5
60-69	99	3	030	80	4	050	1 7	59	3	051	1 7
70-79+	28	1	036	26	1	038	1 1	15	2	133	4 2
Total							2 2				6 0

[a] *Assuming a probability of* 003 *in controls and* 005 in sisters at risk
[b] Assuming a probability of 003 in controls
[c] $p < 01$

The probalities are definitely larger in the mother pedigrees, ranging to 13%, whereas the range is to 5% in the sister pedigrees and 3.6% in the controls. The age-specific risks in this group compared with those of the controls are higher in the premenopausal than in the postmenopausal period. A similar but much less pronounced trend is evident in the other pedigree group. Breast cancer development, therefore, obviously is much more likely at any age in the sisters of patients with affected mothers than among sisters of patients whose mothers were unaffected, or among women with family histories of neoplasms other than those of the breast.

These differences are graphically shown in Figure 1. The cumulative probabilities of breast cancer developing by the end of an age interval are plotted for the two pedigree groups and for two control groups, i.e., the present controls; the cumulative probabilities are also calculated from the morbidity statistics for Connecticut (1935-1962) and New York (1956-1961) presented by LILIENFELD et al. (1972). The sisters at risk in the mother pedigree group have a 32% lifetime probability of breast cancer development compared with a probability of 12% for the sister pedigree group, 8 6% for the present controls, and 7.7% in the New York and Connecticut populations. The two former estimates agree with those estimated from Table 2, indicating the close agreement between the breast cancer rates in present controls and those derived from larger populations. The type of familial pattern of breast cancer thus appears to be another criterion by which the heterogeneity among patients can be reduced.

The mother pedigree group appears to refer to an inherited type of breast cancer, in which predisposition to the disease is vertically transmitted from mother to daughter, such that the daughters have approximately a 30-35% probability of developing the disease during their lifetime. Furthermore, the disease tends to develop early, frequently in both breasts. The pedigrees reported by HANDLEY (1938), WOOD and DARLING (1943), GARDNER and STEPHENS (1950), PAPADRIANOS et al. (1967), CADY (1970), and DMYTRYK (1971), also characterized by early and bilateral disease, appear to be examples of this hereditary type of breast cancer.

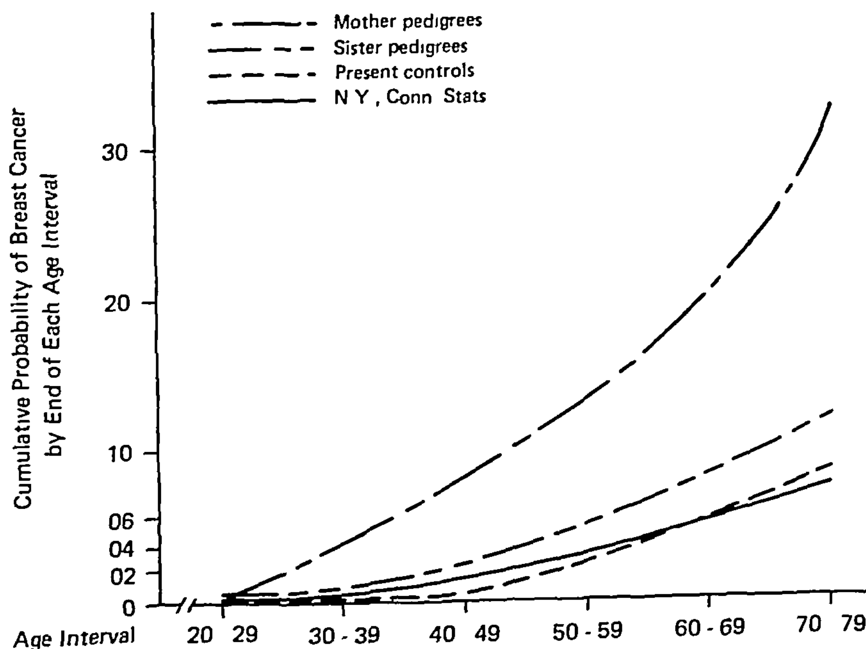


Fig 1 Cumulative probabilities of breast cancer developing by end of age interval

Pedigrees showing later-occurring and unilateral breast cancer have been reported by SMITHERS (1948) WOOLF and GARDNER (1951) and PAPADRIANOS et al (1967); this type also may be included in the present sister pedigree group and perhaps among some of the later occurrences in the mother pedigree group This type perhaps by virtue of its later onset could be influenced by other important risk determinants for breast cancer such as parity and age at first delivery (MacMAHON et al 1973) Preliminary studies have indicated that these two risk determinants have little effect in the mother pedigree group but exert a pronounced effect in the sister group Somewhat similar findings were recently reported by STAVRAKY and EMMONS (1974) They compared premenopausal and postmenopausal women and found breast cancer risk to increase with increasing age at first delivery only among the postmenopausal women

There is evidence therefore of at least two hereditary types of breast cancer that are identifiable when patients are classified according to their age at onset presence or absence of bilaterality and type of family history However additional types are identifiable through the familial occurrences of associated neoplasms such as the familial occurrence of early and bilateral breast cancer in association with soft-tissue sarcomas leukemia and brain tumors (LI and FRAUMENI 1969a b) or the familial association of breast and colon cancer (LYNCH et al 1973) or breast and ovarian cancer (LYNCH and KRUSH 1971) These other hereditary types were identified in 9% of the present families but these occurrences were not confined to any one pedigree group and had no important effect on the results when attention was directed only toward sisters of patients (ANDERSON 1974)

KNUDSON et al (1973) used the two-step mutation model to estimate the fraction of hereditary cases among all patients with breast cancer The primary assumption was that all bilateral cases of breast cancer were hereditary This assumption was based on the analyses of childhood cancers where all bilateral or multiple cases of childhood cancers were considered to be the consequence of a germinal mutation If the bilaterality rate among all breast cancer patients is 3% and 10% in familial cases (ANDERSON 1971) then 30% of breast cancers should be of a hereditary type Not all of the cases would necessarily have a family history of the disease Because the disease might be transmitted through the male line penetrance could be incomplete some carriers would not yet have developed the disease and/or some may have died of other causes before doing so At this institution the fraction of patients with family histories of the disease is about 13-15%

IMPLICATIONS

These results indicate that the heterogeneity among familial breast cancer patients can be reduced by categorizing them according to their age at clinical onset whether bilateral or unilateral type of family history and associated familial neoplasms The reduction in heterogeneity permits the estimation of more realistic estimates of genetic risk which are larger in magnitude than those that emanated from past studies where no attempt was made to reduce heterogeneity The identification of homogeneous groups of patients (and their families) has obvious relevance to providing a homogenous source of experimental material for etiological studies whether clinical genetic viral or epidemiologic However additional criteria for enhancing the homogeneity are still required whether in the form of some type of biochemical viral immunologic pathologic or genetic marker or an endocrine difference

However, no such markers have yet been identified, although two promising possibilities are now available. One is the homology being detected between the base sequences of RNA molecules from extracts of human breast cancer tissue and the DNA of the Bittner mouse mammary tumor virus (AXEL et al., 1972). This homology is evident for some but not all patients (VAIDYA et al., 1974). Another possibility is the proposal by LEMON (1972). He suggested that the enzyme, 16-alpha-hydroxylase, which is involved in the metabolism of 17-ß-estradiol and estrone to estriol, is inherited and influences breast cancer development. Both of these possibilities should be evaluated, particularly in the high-risk group of pedigrees where breast cancer predisposition is following a vertical transmission pattern.

It has already been shown in the present data that a virus is probably not being vertically transmitted through the mother's milk or ovum. Comparisons were made of the disease occurrence in maternal and paternal grandmothers of patients, maternal and paternal aunts, and in sisters of patients when prior disease occurred in a maternal or paternal aunt. The rationale for these comparisons was that if a virus were being transmitted through the milk or ovum, the disease would be more prevalent in the maternal than in the paternal side of the family. However, the comparisons provided no evidence of any differences in disease frequency whether the disease followed a maternal or paternal line of descent. For all comparisons, the frequency of breast cancer was 6.8% in the maternal line and 7.2% in the paternal line (ANDERSON, 1974). Although these findings suggest that a virus is not transmitted through the ova or milk, they do not, however, rule against the possibility of a virus in human breast cancer; however, if a virus is involved, it must be ubiquitous and/or integrated into the DNA of either the male or female parent.

The present results thus point to different hereditary types of breast cancer. Genetic heterogeneity implies different etiological mechanisms. If the hereditary types account for 15-30% of all breast cancer cases (KNUDSON et al., 1973), then the remaining 70-85% of the cases would also appear to involve several different etiologic mechanisms. But the major effort in the past studies, whether genetic, viral, epidemiologic, immunologic, or hormonal, has been to explain the disease on the basis of a single etiologic factor. Perhaps more progress would be possible in understanding the vagaries of breast cancer if this simple hypothesis of a single disease were replaced by the concept of a heterogeneous disease. The hypothesis of a single disease has certainly obscured the identification of a genetic basis in past genetic studies. It is likely also helping to obscure the identification of other etiologic agents.

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Chapter 3
RNA Tumor Viruses and Breast Cancer

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Over the past 60 years a considerable amount of information has been amassed concerning the role of viruses in naturally occurring cancers In particular the group of viruses known as the RNA tumor viruses has been implicated in a variety of cancers in a wide spectrum of animals In addition to the presence of the virus numerous genetic hormonal and environmental factors play an important permissive role in the development of many neoplasias Although the presence of RNA tumor viruses in humans or their involvement in human cancer remains as yet unproven information from experimental systems is clearly consistent with this possibility Recent advances in molecular and cell biology and immunology have been applied to the search for a human virus These studies have provided evidence for the possible presence of these viruses in human tumors In this presentation we would like to review the recent evidence concerning the unique biologic characteristics of the RNA tumor viruses as well as the methods currently being utilized in attempts to detect their presence in experimental animal and human systems Consideration will also be given to the relevance of these findings to the human breast cancer problem

The RNA tumor viruses as a group are the only known RNA-containing viruses with proven oncogenic potential In contrast to the known oncogenic DNA viruses such as the herpesviruses which either destroy cells during a lytic cycle of infection or transform cells without virus production the RNA tumor viruses are generally oncogenic in their host of origin and multiply in the absence of any cytopathic effect on the host cell Those RNA tumor viruses associated with the induction of lymphomas leukemias and sarcomas are called type-C viruses based on their morphology by electron microscopy (BERNHARD 1960) They have been isolated from almost every animal class and are structurally biochemically and biologically similar although they can be distinguished one from another by sensitive immunologic and biochemical techniques The RNA tumor virus associated with the induction of mammary cancer in mice the mouse mammary tumor virus (MMTV) exhibits a morphology distinct from the C-type viruses and is the prototype of a group of viruses termed the B-type viruses

Structurally the RNA tumor viruses are spherical particles 80-130 nm in diameter possessing an outer lipid-containing membrane Within the particle is an electron-dense core consisting of an RNA-containing nucleoid centrally located in the C-type viruses or eccentrically located in the B-type viruses Large stable surface projections 5-10 nm in length are quite evident on B-type virions while considerably shorter projections are seen on C-type viruses (SARKAR et al 1971) All the RNA tumor viruses have the following properties: (1) maturation is by budding through the cell membrane (type-C viruses bud with an incomplete nucleoid while the type-B viruses bud with a complete nucleoid) (2) a density of 1 15-1 19 g/cc in sucrose gradients (3) a high mole-

cular weight RNA with a sedimentation coefficient of 60-70S and a molecular weight of about 8 X 10^6 daltons as the viral genome, and (4) possession of an enzyme termed "reverse transcriptase," which transcribes DNA from an RNA template. This enzyme converts the viral genetic information, which is in the form of RNA, into a DNA which can be integrated into the host cell DNA This allows for the viral information to be vertically transmitted in an unexpressed form as an integral part of the host cell genome.

The fact that oncogenic RNA viruses possess a large single stranded high molecular weight RNA and a "reverse transcriptase" has led to an in vitro test for the simultaneous detection of both characteristics (SCHLOM and SPIEGELMAN, 1971; SCHLOM et al., 1972) It was shown that the small initial radioactive DNA product of the viral reverse transcriptase reaction is found complexed to the 70S RNA template. The radioactive DNA: viral RNA-complex sediments in the 60-70S region of a glycerol gradient and bands at the density of RNA in cesium sulfate gradients. Therefore, if a radioactively labeled viral DNA product from an unknown sample was found complexed to a 70S RNA template, evidence would be presented for the presence of an RNA tumor virus in the material being examined. There have been a number of reports on the presence of RNA tumor virus-like particles in samples of human milk. It has subsequently been shown that human milk contains particles with a density of 1.16-1.19 g/cc, which have a reverse transcriptase (SCHLOM et al., 1971). The demonstration of the viral origin of this reverse transcriptase activity in human milk is of obvious importance because it has been shown that the causative agent of murine mammary tumors, the mouse mammary tumor virus, can, at times, be detected in mother's milk regardless of the mode of transmission of the virus (BENTVELZEN, 1972) Studies utilizing the simultaneous detection test were undertaken to see whether these particles isolated from human milk contained 70S RNA, and whether this RNA was used as a template in the reverse transciptase reaction. Numerous human milk preparations have been examined with this assay, and several were shown to be positive (DAS et al., 1972; FELLER and KANTOR, 1972, GERWIN et al., 1973; SCHLOM et al., 1971, SCHLOM et al., 1972). It was observed that women differ both in their content of particles with reverse transcriptase and a 70S RNA and furthermore, that different samples from an individual woman differ from day to day (SCHLOM et al., 1972). Similar studies have also been conducted with human malignant breast tumor tissues. These studies were facilitated by applying the technology developed for the preparation of subviral particles of MMTV from murine breast tumors to human malignant breast adenocarcinoma tissue (MICHALIDES et al., 1975b). Positive results were found in several malignant tumors, but no reverse transcriptase activity was observed in benign breast tumors. These studies provided evidence for the presence of particles in certain human cancers with biochemical properties similar to those of the RNA tumor viruses

A number of studies have also been performed to determine whether human breast tumors contain genetic information which is homologous to that of a putative or known viral agent (e.g., to the particle found in human milk or to a known animal RNA tumor virus). These studies utilized the radioactively labeled viral DNA product of the reverse transcriptase reaction, in molecular hybridization experiments, to detect any nucleic acid homology with the RNA of human malignant breast tumors Positive evidence has been reported that the RNA of some human breast tumors contain nucleic acid sequences related to MMTV (AXEL et al., 1972; VAIDYA et al., 1974). In addition, positive hybridization has also been reported to another RNA tumor virus, the Mason-Pfizer monkey virus (MPMV) (COLCHER et al., 1974) However, benign tumors and normal tissues were always negative. Additional studies will be required to

determine the significance of the presence of viruses in human breast tumors Future studies will incorporate recently developed sensitive and specific radio immunoassays for RNA tumor virus proteins and should aid in the detection of low levels of these viruses if present in human tumors Recently YEH et al (1975) reported finding antigens related to the major structural protein of MPMV in 8 out of 18 human breast tumors using a sensitive radioimmunoassay for this protein Normal tissues and human tumors of other organs were negative

If it is true that a virus related to either MMTV or MPMV operates in the genesis of human breast cancer it is of utmost importance to understand the role of the mouse mammary tumor virus in the genesis of murine breast cancer In this animal model system many important experiments are feasible and can be well controlled Furthermore breast cancer in the mouse represents the only animal system where a virus has been clearly shown as a causative factor in the development of the disease

Induction of mammary carcinomas in mice is a complex phenomenon in which hormonal stimulation genetic background and the presence of a mouse mammary tumor virus all play a role Various mouse strains have been shown to have their own strain-specific MMTV variant with its own characteristic expression virulence host range and mode(s) of transmission (BENTVELZEN 1972) The mode of transmission of any particular MMTV variant appears to be determined by both the host genotype and the nature of the MMTV variant There are two known modes of transmission of MMTV in mice The first mode is vertical transmission where the MMTV variant is present as a provirus (viral information in the form of DNA) in the cellular genomes of all cells deriving from the zygote The cellular DNA of all somatic cells of the mouse therefore contains all the proviral sequences of this vertically transmitted MMTV variant For example the MMTV variant in the mouse strain GR is believed transmitted in this way The second mode is horizontal transmission where the MMTV is introduced into a mouse strain by infection The most common infection route is through milk via nursing Occasionally MMTV may be transmitted by male seminal fluid to females which in turn can transfer the virus to their progeny via milk Other modes of horizontal transmission are also possible In the case of horizontal transmission one might expect the cellular DNA of only the infected cells to contain the proviral sequences of the horizontally transmitted MMTV variant

The mammary tumor incidence in mouse strains carrying only the vertically transmitted MMTV is usually low (less than 20%) and the tumors usually appear late in life (later than 13 months) In contrast the mammary tumor incidence in mouse strains carrying a horizontally transmitted MMTV is usually high (greater than 80%) and tumors usually arise early in life (six to nine months)

Utilizing sensitive and specific molecular hybridization experiments it has been possible to demonstrate that there is greater than 95% nucleic acid sequence homology between mouse mammary tumor viruses isolated from primary mammary tumor cell cultures of the mouse strains RIII GR A and C3H (MICHALIDES and SCHLOM 1975) However a comparison of the genomes of the milk transmitted MMTV and the vertically transmitted MMTV of the mouse strain C3H has revealed that these two viruses are only 75% similar (MICHALIDES and SCHLOM 1975) These findings support previous studies that there are at least two classes of MMTVs It is significant that newborn C3H mice which have been foster-nursed on mice which contain no overt milk-borne MMTV and which contain the vertically transmitted virus have a low incidence of mammary tumors and the tumors appear late in life Normally these mice would

exhibit a high incidence and early appearance of mammary tumors if nursed by their own mothers. It will be of interest to determine whether the additional genetic information carried by the horizontally transmitted MMTV is responsible for the higher tumor incidence and whether this information, if isolated, will prove to be a useful probe to detect the presence of RNA tumor virus genetic information in human brest tumors.

There are other RNA tumor viruses whose roles in the induction of breast cancer in animals are being studied (see Table 1). One of these, the Mason-Pfizer monkey virus, was isolated from a mammary carcinoma of a rhesus monkey (CHOPRA and MASON, 1970; MASON et al., 1972). Although it has not been shown as yet to possess oncogenic potential, there is evidence, as mentioned previously, for the presence of genetic information and antigens related to this virus in human breast cancers, but not in benign tumors, normal tissue, or other types of human tumors (YEH et al., 1975; COLCHER et al., 1974). Another RNA tumor virus is the rat virus, R-35, which was isolated from a transplantable mammary adenocarcinoma of the rat (AHMED et al., 1972). Its role in the development of mammary neoplasias is unclear at this time. The endogenous guinea pig virus is of interest because it resembles MMTV both morphologically and biochemically (MICHALIDES et al., 1975a).

The studies which we have summarized provide evidence that RNA tumor virus-like particles or viral components can be detected in certain human breast tumors. The experimental approaches utilized in these studies have taken advantage of the biological and biochemical properties of known RNA tumor viruses. It must be emphasized that these findings in human tumors are still preliminary, awaiting both the isolation of a putative RNA tumor virus and its propagation in sufficient quantity so that it can be fully characterized. For the present, it is important to continue not only studies on human material for the presence of an RNA tumor virus but also studies which are aimed at understanding the mechanisms by which analogous viruses cause tumors in experimental animal systems.

Table 1 Viruses possibly associated with breast cancer

1. Mouse mammary tumor virus (MMTV)
   a) Etiologic factors in numerous mouse strains
   b) Nucleic acid homology with RNA of human mammary adenocarcinoma
2. Mason-Pfizer monkey virus (MPMV)
   a) Isolated from mammary carcinoma of rhesus
   b) Nucleic acid homology with RNA of human mammary adenocarcinomas
   c) Antigen of major structural protein found in human breast tumors
3. R-35 rat virus
   a) Isolated from transplantable mammary adenocarcinoma of the rat
   b) Transforms rat mammary cells in vitro
4. Guinea pig virus
   a) Induced by BrdU treatment of guinea pig embryo cells
   b) Similar to MMTV both morphologically and biochemically

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Chapter 4
Utilization of Diagnostic Techniques for Cancer of the Breast – Early Diagnosis*

PH STRAX

Early diagnosis of breast cancer is the only method with proven potential for lowering the death rate from the disease Early diagnosis means, in a practical way, detection of *preclinical cancer* - *finding* the cancer before it would ordinarily be detected in the normal course of events. This involves mass screening of apparently well women.

BACKGROUND

When we consider that breast cancer is the most common cancer confronting the American and Canadian woman with 1 in 14 getting the disease and that it is the number one killer of women aged 40-44 in the United States and in those aged 35-50 in Canada, its importance is obvious It is the most common cause of cancer death at any age The breast is the site of 28% of all cancer in women, making it the most common site of all cancer by far. It is particularly distressing when we realize that in spite of surgical radiotherapeutic, and chemotherapeutic advances, the mortality rate from this disease has not changed significantly in 40 years.

It is well known that detection of breast cancer at a time of no nodal involvement - presumably clinically localized - carries with it an 85% 5-year survival. When nodes are involved, the figure drops to 53% or even lower when two or three glands show metastases. At the present time only about 25% of breast cancer patients are alive and free of disease ten years after diagnosis. Perhaps the reason for this poor showing is that breast cancer is 95% of the time diagnosed by the patient. The woman herself finds her lump and brings it to her physician' attention after more or less delay. Perhaps what is needed is to have physicians take over breast cancer detection, i e., to get a woman in for examination before she is aware of a problem, hopefully when the cancer is in an earlier stage.

The Health Insurance Plan Study

These facts led directly to the initiation in 1963 of the program of mass screening for breast cancer conducted by the Health Insurance Plan of Greater New York under contract with the National Institutes of Health (SHAPIRO et al., 1973). The project was set up to answer two premises:

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1 Would mass screening for breast cancer using both clinical examination and mammography result in a lowering of the mortality rate; in other words would such screening result not only in longer survival, but in actual saving of lives as indicated by a lowering of the death rate?
2 What would be the contribution of mammography in such mass screening - or could mammography indeed contribute enough to make it worthwhile to add to a screening program?
Sixty-two thousand women aged 40-64 were chosen at random from 23 of the Health Insurance Plan Medical Groups They were chosen as 31 000 pairs with 31 000 in a study group and an equal number in a carefully matched control group The study group was offered an initial examination and three subsequent annual examinations The control group received its usual medical care and was followed through records Two-thirds of the study group responded to the invitation and received independent clinical examination and mammography performed by a modified Egan technique using slow industrial-type film with low kilovoltage and only inherent filtration One-third of the women did not respond and were followed through records as part of the study group not screened A detection rate of 2 72 cancers per 1000 was found in the examined women compared to 1 86 in the control

In a 7-year follow-up 108 deaths from breast cancer occurred in the control group compared to 70 in the study group - only two-thirds of whom had actually been examined The one-third reduction in mortality is persisting after 8 years of follow-up There would thus seem to be a rational basis for expanding the concept of mass screening for early detection of breast cancer as a means of saving lives of women afflicted with this disease

It was also found that under the conditions of this study mammography contributed substantially to the yield In this program one-third of the cancers would not have been detected if this modality had been omitted At the same time the importance of clinical examination in screening was emphasized Two-fifths of the cancers would not have been found if clinical study had been omitted Both modalities were essential for the yield These figures are considerably different from those obtained in general clinical practice when both modalities have a high degree of correspondence - well over 80% This is probably because under usual medical conditions late breast cancer is ordinarily found Early cancers apparently are more often detectable by only one modality

This is pointed up by the fact that 75% of those found on clinical examination alone were free of nodal involvement and 79% of those found on mammography alone had negative nodes To date only 1 woman out of 44 whose cancer was found on mammography alone had died

When statistical methods were applied to analyzing the probability of a woman dying from any cause when she had had a proven breast cancer - the case fatality rate - and an average statistical lead time of 1 year was assumed it was found that the major factor in reduction of death rate was the low incidence of cancers detected in the screening process especially through mammography

Another important finding was that 15% of the cancers found in the study developed within 12 months after an apparently negative examination These so-called interval cancers had the same rate of nodal involvement as the control group In subsequent screening programs this factor needed further attention

It was also noted that the entire reduction in mortality was concentrated in women over the age of 50. Those under 50 showed no such improvement. Mammography is less effective in younger women when the breasts are more glandular and more dense on the mammograms.

In considering further mass screening efforts in the general population, these facts led to three areas of improvement:

1. Emphasis needed to be placed on breast self-examination. It was this procedure that could detect the interval cancer in an earlier stage. The woman herself needed to become our ally in detection of breast cancer. It was felt strongly that this procedure needed to be taught to women directly on a one-to-one basis. The pamphlets distributed over the years by the cancer societies were just not enough.

2. Improvement in mammography was needed, particularly to produce mammograms with greater resolution and contrast in the younger women. Such improvement became available in the molybdenum anode tube x-ray machines, the first of which was the senograph, developed by Charles Gros of Strasbourg and his associates. This produced a considerable improvement in detail, resolution and contrast when compared with the conventional mammogram.

Another form of improved mammogram is the xerogram, developed by WOLFE (1972) in which a charged selenium plate is used instead of film. Xerography depends for its success primarily on the enhancement of edges characteristic of the process. Microcalcifications with their definite edges are clearly delineated. Cancer masses which may have poor edges may actually be poorly visualized. The value of the grey scale of film in depicting masses is absent. The process is enhanced by the harder rays from a tungsten anode and higher kilovoltage than is used with film. Conventional x-ray equipment - provided compression is added - can produce mammograms with the xerographic process which are superior to so-called conventional mammograms made with the EGAN (1962) technique. Newer film techniques with special mammographic equipment are capable of greater efficiency and lower cost than xerography, especially in mass screening. There is a difference of opinion on the relative value of this display medium compared to film. The newer films used today with vacuum cassettes as developed by PRICE and NATHAN (1975) and others in England are leading to markedly enhanced film mammograms with considerably reduced radiation dosage, below that of xerography. Many experienced workers prefer the improved films, especially for mass screening. Both the enhanced film mammogram and the xerogram are capable of much improved results over the type of conventional mammogram used in the H.I.P. study.

3. The thermogram, introduced by RAY LAWSON (1957) of Montreal, is an additional modality that can alert the clinician to the possibility of abnormality in one breast compared to the other. Increased heat is often associated with the malignant process. Some 20% of women examined, however, have a hot thermogram in one breast without abnormalities on other modalities; furthermore, such areas of increased heat do not necessarily depict the site of a cancer. In fact some 30% of cancers do not show increased heat. It is thus felt by many that thermography is not an anatomical detector of cancer but an alerter of a physiological disturbance which may be a malignant process, but which requires corroboration by other methods. A hot thermogram may also be a high risk marker indicating increased possibility of a clinical cancer developing.

The Guttman Institute

These considerations led to the development in 1968 of the Guttman Breast Diagnostic Institute in New York City with the basic objective of developing a mass screening approach that would be feasible and could reach the general population of women The two major problems to be solved were how to develop a practical economical and efficient method that could produce a satisfactory yield of earlier cancers and how to motivate women to accept such a procedure

A tandem approach to breast cancer detection has been developed that has offered the best opportunity for greater yield (STRAX 1971) It consists of the following:

1 An interview which is usually conducted by volunteers Demographic data menstrual genital breast and family history are obtained

2 Clinical Examination: This phase is under the supervision of a physician well versed in this procedure Data are put on special cards Breast characteristics are recorded Clinical impression as well as recommendation for further study is made At this examination the woman is taught the technique of self-examination and urged to follow through every month

3 Mammography Soft tissue x-ray examinations of the breast are performed with the senograph This special apparatus developed for breast study produces mammograms of a high technical excellence rapidly (about 10 women per hour) with low radiation dose (2-2 5 rads per exposure) by using nonscreen film By using special rare-earth screens and matched films the radiation dose has been brought down to under 0 3 of a rad A device is being built which will enable a technician to use this improved technique efficiently and quickly with a reduction in cost of film and operation of about one-half Two views are made of each breast the cranio-caudal and the medio-lateral Data are recorded on special cards with details of breast type summary of findings and recommendation for further study

4 Thermography: This graphic representation of the infrared emanation from the breast is made with an AGA Thermovision This device produces an instantaneous photographic image on 70-mm photographic film Impression of heat pattern and recommendation for further study are made Since an area of increased heat does not necessarily correspond to the location of a cancer thermographic findings are used to alert the clinician to findings on other modalities

Since clinical examination mammography and thermography detect different facets of breast cancer and since these methods are complementary, all three are used on all patients over 35 Under 35 the x-ray is omitted

Training of paramedical personnel supported in part by the New York City Division of the American Cancer Society is directed to several areas Several of the Institute s personnel have been trained as prescreeners in clinical examination of the breast Several nontechnicians have been trained to do thermography Three of the x-ray technicians have developed considerable skill in reading mammograms as prescreeners All examinations are done independently so that the contribution of each modality may be assessed

With efficient logistics and concentration of studies in one room so that all are done under cooling conditions the entire examination can be done in less than 20 min at a cost of about $ 20 to the Institute No charge is made to screenees

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Results at Guttman Institute

Of 80,422 examinations made in 1971-1974, 40,341 were intial studies and 40,101 were subsequent examinations. Of 3367 recommendations for biopsy or aspiration, 1910 were done and 478 cancers were found

Value of Periodic Examinations

On initial examination, the number of prevalent cancers present was high, depending on such factors as self-selection and age of women Because cancers had been present for varying lenths of time, only half of the cancers were free of nodal involvement. On subsequent examination, the number of incident cancers, which had become detectable since the previous exam, was much less, but the majority had no nodal spread (Table 1).

Table 1. Negative axillary nodes

	Exams	No of cancers	Rate/1000	No of cancers	%
Initial exams	40,341	374	9 4	185	49%
Subsequent exams	40,101	104	2 6	66	63%

Value of the Tandem Technique

The tandem technique, using clinical examination, mammography, and thermography gave the highest yield of cancers because some were detectable on only one modality. Those found on one modality only had a higher percentage of negative modes.

1. Of the 478 cancers found, 437 were detected on screening modalities and 41 were interval cancers found by the women themselves within a year of a negative examination. They are not included in the tally below.

2. Thermography, when abnormal, alerts the physician to the possible presence of cancer in a stage not yet detectable by palpation or mammography and suggests more frequent examinations in order to localize it earlier. Since a positive thermogram alone is never the basis for biopsy, it is also not represented in the data below

3. Negative nodes are included in the following data only when proven The surgical procedures in 12% of the cases did not include axillary dessection, so that the true percentage of negative nodes may be higher. (Table 2).

Table 2 In 437 cancers (excluding 41 "interval" cancers)

Cancers detected by	All Cancers No	%	Negative Axillary Nodes No	%
Mammography only not palpable	75	17%	52	69%
Clinical exam only not on x-ray	121	28%	75	62%
Both felt on palpation and seen on mammogram	241	55%	98	41%

Value of Breast Self-Examination (BSE)

Since 95% of breast cancer is first detected by the women themselves indoctrination into BSE is vital as a first step in screening Women can be taught (and should be taught by the physician) to detect small lesions of 1 cm or less in their own breasts BSE is especially important to detect interval cancers When BSE indoctrination is part of the screening process interval cancers are detectable with a high degree of no nodal involvement

Of the 41 interval cancers 27 or 66% had no nodal involvement

To reach the low-income woman it is necessary to go into the communities and screen on an outreach basis Mobile equipment is used The four-part technique used is the same as that described above However mammography may be performed with a 70-mm device (STRAX and OPPENHEIM 1968) that uses ordinary house current is transportable and is capable of examining up to 20 women per hour with a radiation dose of 1-1 5 rads per exposure This device is being optimized to permit the use of large full-scale films also at the rate of 20 women per hour but with radiation doses of under 0 5 rad per exposure The device will also be transportable and use ordinary house current

A highly maneuverable 26-foot self-contained mobile unit (STRAX 1972) is also used in community screening This van contains facilities for the same four-part examination as at the fixed facility with the same equipment It is manned by trained allied health personnel and is capable of examining 70 women per day It maintains a close tie with the fixed facility for scheduling and follow-up

It should be noted that the Guttman Institute accepts all women whether symptomatic or not No attempt is made to weed out those with obvious problems The percentage of women with symptoms of lump pain or discharge is high Until recently a 1-month backlog was maintained as the only device to coax women with frank disease to see their private physicians In recent months the backlog has increased to 3-4 months and an expansion program is in progress to shorten the waiting period

The data presented therefore include a high preselection of women The percentage of occult cancers would be much higher if only asymptomatic women were screened or if there was a cut-off at age 35 Guttman Institute however feels that its function is the practical one of examining all women who seek its services regardless of age or breast condition We consider the screening process as an extensive educational program As a result of the success of the Guttman Institute 28 other programs of similar nature have been set up in the United States under the auspices of the National Cancer Institute and the American Cancer Society All of them are finding earlier cancers with a high rate of no nodal involvement All of the programs have been set up to examine up to 10 000 women for a period of 5 years and then to have follow-up study for 5 years after that Only asymptomatic women are screened at these centers

Conclusion

1 Women can be motivated to accept the complete examination At the Institute where an open-end operation is in progress the number of applicants has been increasing steadily It is common knowledge that in those programs which are limited in numbers a waiting period of 6 months to a year is common Motivation of low income women however has been lagging Even at the Guttman Institute where much effort has been expended in stimulating this group success is limited to only

10-15% of screenees. Only the out-reach program, where examinations are performed directly in communities, has been able to reach this sector.

2. A gratifying proportion of breast cancers has been found in earlier stages with no nodal involvement and presumably improved survival and perhaps curability. Both clinical examination and mammography have contributed to the improved picture.

3. The educational process involved in screening is important, not only in alerting the female population to accept screening, but in indoctrinating the medical profession into the value of the screening process for detection of earlier breast cancer.

4. The value of allied health personnel in all facets of the screening process has become apparent. Clinical examination by a nurse or other paramedical personnel is readily accepted and has demonstrated its value to the supervising clinician. The potential of such personnel in prescreening mammograms and thermograms is also of great interest.

5. The indoctrination into breast self-examination results in detection of the interval cancer in an earlier stage with lowered axillary nodal involvement than in usual medical practice.

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Chapter 5
Human Breast Cancer in Culture

G L. TREMPE

INTRODUCTION

Since many techniques in immunology virology biochemistry and biophysics depend upon the availability of homogeneous populations of human tumor cells, the establishment of permanent homogeneous tumor cells lines is a useful approach in experimental human cancer research However the cultivation of human breast tumor cells remains a challenging problem This paper presents our experience in growing these tumor cells in vitro

COLLECTION

Maximum cooperation must exist between the surgeon the pathologist and the technicians in order that the excised specimen is kept sterile and is cultured within one to two hours Since many tumors removed at surgery are very small our cultivation attempts have often been hampered by the limited size of the specimen Chances for successful growth increase with the number of live tumor cells available

METHODS OF PREPARATION AND INITIAL CULTURE

The techniques of preparation and initial culture are summarized in Figure 1 Under sterile conditions the tumor specimen freed from fat and necrotic tissues was washed in medium and minced into pieces of approximately 1 mm^3 size After several additional washings the solid tumor specimen was treated as follows:

1 One or two pieces randomly chosen were sent to pathology for confirmation of the diagnosis
2 The cells spilled during mechanical separation were washed and cultured separately
3 Fragments were treated with trypsin and/or collagenase at 37^o and the recovered cells seeded in culture flasks after several washes
4 Other fragments were explanted directly in tissue culture flasks and a thin layer of medium added to produce maximum attachment and migration

Cells obtained from pleural effusions were recovered by centrifugation and seeded after minimal manipulation in fresh medium

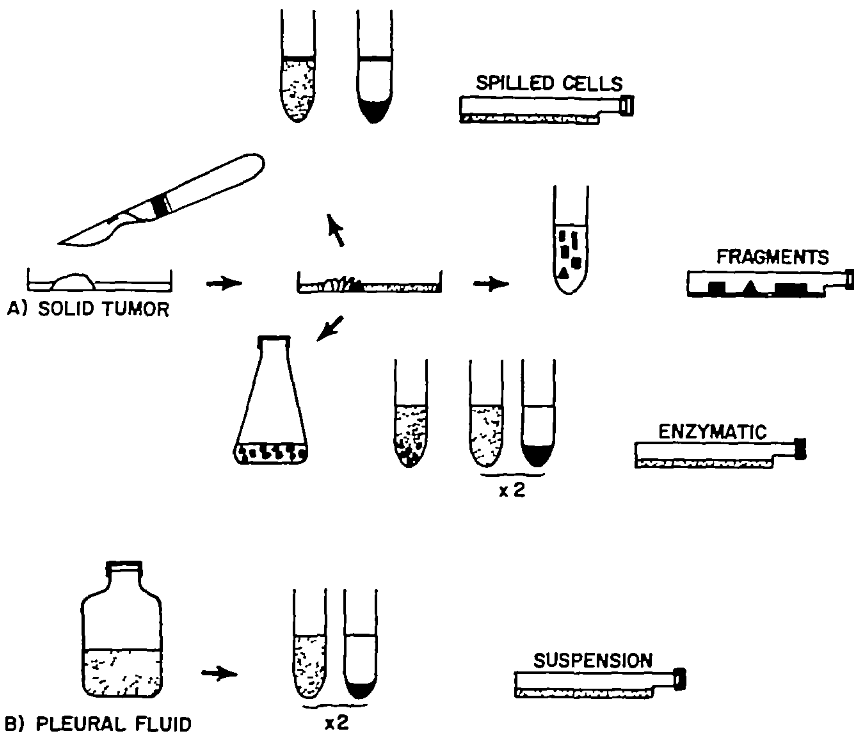


Fig 1. Techniques of preparation and initial culture of human breast tumor cells Cells obtained from solid tumors after mechanical or enzymatic dissociation are cultured separately Fragments of solid tumor are explanted directly Cells collected from pleural fluid are washed and cultured readily (Adapted from Lasfargues, 1975)

Samples of free floating cells obtained after various treatments of the solid tumors or directly from pleural fluid were sent to cytology section of the department of pathology. Specific cytologic criteria of malignancy, based on experience with exfoliated tumor cells, were applied to these specimens after Papanicolaou staining. After serial passages in vitro, cells considered established as a permanent cell line were re-evaluated by the same cytologic criteria.

NUTRITION

Different media were used during the initial cultures as well as later for the permanent cell lines. The most successful medium was the Eagle's MEM supplemented with fetal calf serum and/or human serum with a mixture of insulin, hydrocortisone, and prolactin, each at a concentration of 5 mg/ml. All cultures were grown in glass T-flasks. Penicillin, 100 units per ml, and streptomycin, 100 mg/ml, were added to all media. All cells were tested regularly for mycoplasma contamination and were found to be free of this contaminant.

IMPORTANCE OF TUMOR MORPHOLOGY

Human breast tumors are known to vary widely in morphology ranging from a solid cluster of tumor cells to a very fibrotic acellular tumor. The

great majority of the human breast tumors are of the scirrhous type This explains partially the lack of success encountered in establishing a large number of permanent human breast cancer cell lines The majority of the tumors are necrotic or dead and the surviving cells are mostly originating from the fibroblastic understructure This explains some of our own results which are summarized in Table 1

Table 1 Malignant human breast solid tumors

Predominant histologic type	#	% Successful primary epithelial outgrowth	# Permanent cell line
Infiltrating duct carcinoma (mostly scirrhous)	54	12 9 (7/54)	0
Other (medullary lobular mucinous tubular papillary)	14	48 8 (6/14)	0

Relation between pathologic diagnosis and successful culture in solid breast tumor Note the relative success in growing cultures with epithelial features with solid breast tumors of the nonscirrhous type although no cell line could be established permanently from any solid tumor

Of 68 solid human breast tumors 54 showed a scirrhous component While nearly 50% of epithelial outgrowth was obtained with the nonscirrhous type of tumor only 7 out of 54 scirrhous tumors showed a growing population of epithelial cells In all those cases however, within a short time the fibroblastic component would literally strangle the epithelial outgrowth which would soon be found floating curled up in the culture flask No permanent cell line could be established from solid tumors during 3 1/2 years Many of the primary cultures with a mixed population of epithelial and fibroblastic cells could be used in biologic studies which we will discuss briefly later on

The enzymatic treatment with trypsin or collagenase produced a better yield of cells but unfortunately more fibroblasts were released from the connective tissue treated and these normal cells slowly outgrew strangled and released the epithelial cells into suspension

Table 2 reports the results obtained with malignant effusions from patients with breast cancer Thirty-seven malignant effusions were used to initiate cultures Thirty-six patients were in fact patients with recurrent disease and the cytologic study confirmed the presence of malignant cells in their pleural fluid The original histologic

Table 2 Metastatic pleural fluid as a source of tumor cells in patients with breast cancer

Original Predominant histologic type	#	% Successful primary epithelial outgrowth	# Permanent cell line
Infiltrating duct carcinoma (mostly scirrhous)	31	77 4 (24/31)	1
Others	5	100 (5/5)	0
Unknown[a]	1	100 (1/1)	1

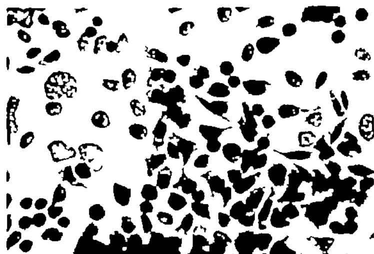
Note the significant results in culturing epithelial tumor cells obtained from sedimented pleural effusion and the negative relation with histologic diagnosis

[a] Histologic diagnosis unestablished before post-mortem highly anaplastic breast tumor

types of these patients are shown. One patient did not have a histologic diagnosis prior to hospitalization. She showed up with bilateral malignant effusions, but the origin of the primary tumor was impossible to specify until she died. At that time, the autopsy report a highly anaplastic breast tumor over an ovarian carcinoma. Cytologic criteria applied to the growing cultured cells favored also the breast tumor origin. Although the original histologic type was scirrhous in 31 cases, we did succeed in obtaining cultures with predominant epithelial feature in a large majority of our trials, in fact in 77%. With 6 other cases, 100% of the initial cultures yielded an almost pure population of epithelial cells. These primary cultures gave two permanent cell lines (see later). The use of pleural fluid eliminated, therefore, two of the main difficulties which we have encountered in cultures obtained from solid breast tumors: fibroblast and necrosis. The prevailing fast growing fibroblasts which form the connective tissue understructure of any solid tumor are practically nonexistent in a pleural effusion. Moreover, the tumor cells present are generally 50-100% viable This contrasts significantly with a tumor cell viability of 5-50% in the majority of the breast malignant tumors which we have handled Another advantage is that large numbers of tumor cells can be pooled out of the effusion usually as single cells or clusters. Finally, the possibility of obtaining multiple effusion samples from the same patients over a relatively long period is priceless for in vitro and in vivo studies (CAILLEAU, 1975). Mesothelial-like cells tend to contaminate many effusions. These cells are usually large and settle very rapidly on the surface of any tissue flask. They slowly invade and remove the tumor cells attached on the flask as fibroblasts do. Fortunately, their rate of mitosis is low and they reattach poorly after trypsinization. Leukocytes, and in particular lymphocytes, can also contaminate malignant effusions although these cells will attach poorly and tend to divide into suspension.

PERMANENT CELL LINES

SK-BR-3 is one of the permanent cell lines established in our laboratory (FOGH and TREMPE, 1975). The tumor cells are generally recognizable Epithelial tumor cells tend to clump or grow as clusters at the surface of the flask; sometimes they float. Since some of the tumor cells from pleural effusions remain in suspension for days before attaching to the tissue culture flask, we have used this ability to separate such cells from the contaminating mesothelial-like cells which attached rapidly to the flask surface. SK-BR-3 was established from a malignant pleural effusion of a patient known to have metastatic breast cancer. Figure 2 at the top shows the H and E staining of cells growing on a glass slide. These cells tend to pile up and do not show contact inhibition. The bottom picture shows living cells in culture through intergerence contrast microscopy or Nomarsky technique. Pleural fluid was aspirated from a 43-year old caucasian female patient, blood type A Rh positive on November 19, 1970. The examination by the cytology departments at New York Hospital and Memorial Hospital gave strong indications of a malignant neoplastic type of adenocarcinoma. The patient expired two weeks later and the autopsy revealed a poorly differentiated adenocarcinoma of the left breast with multiple tumor giant cells containing mucus-like substance in the cytoplasm. There were metastases to the right breast, bones, and pleura. Approximately, two liters of very bloody fluid was received in our laboratory. Separation of erythrocytes from tumor cells was incomplete even after several hypotonic treatments of the pellets, obtained after initial centrifugation of 300 cc of fluid. The cells were seeded in a MEM medium with 10% fetal calf serum, 10% human serum and hormones; the cultures were incubated for 10 days without medium change. At the time, small groups of epithelial cells were attached to glass. Many cells were also observed floating in the cul-



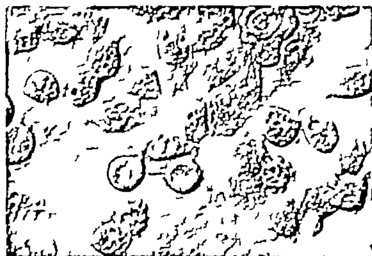


Fig 2 SK-BR-3 breast tumor cell line P 28 H E and interference contrast microscopy (Nomarsky) x 800

ture fluid These were transferred to new culture containers in which approximately 10-20% attached, and viable cultures were therefore established After five weekly transfers by this method human serum and hormones were omitted from the medium and half confluent cultures were trypsinized for the following culture passages: first every three weeks later each week The transfer factor increased from twofold to fourfold Floating cells although malignant and epithelial according to cytologic criteria were purposely discarded during further medium change and culture transfers in order to select a glass attached cell population in preference to a suspension culture This cell line is now being carried up to more than 50 culture passages It has been frozen in liquid nitrogen at different passages

SK-OV-1 is another permanent cell line obtained from a pleural effusion Figure 3 (FOGH and TREMPE 1975) Clear pleural effusion was obtained on December 22 1970 from a 65-year old caucasian female patient blood type O Rh positive The presence of malignant cells was established by cytologic examination The patient from which this cell line derived was assumed to have an ovarian cancer but postmortem studies favored a highly anaplastic breast tumor Four months after initiation of the primary culture the first transfer was made Within a few months this cell line was transferred weekly at dilutions of 1:3 or 1:4 The highest present passage level is 35

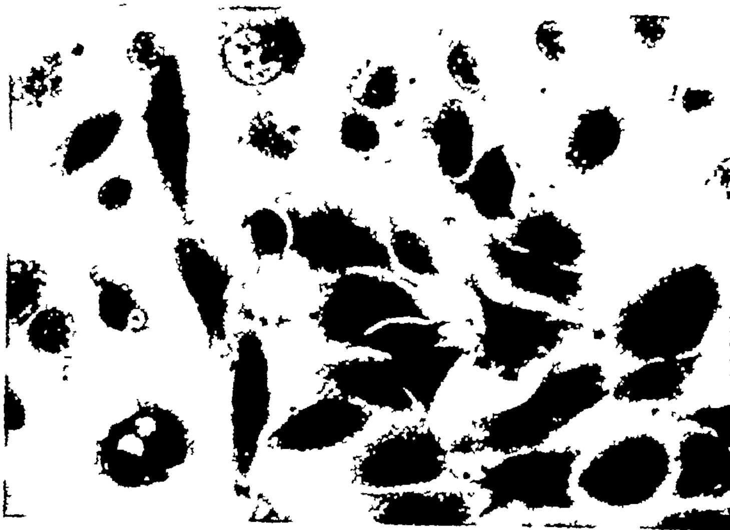




Fig 3 SK-OV-1, breast cancer cell line, P 16, H E and interference contrast microscopy (Nomarsky) x 800

Characterization of these two lines has been carried on further. The SK-BR-3 line expresses the blood group A at the cell surface as well as inside the cytoplasm.

Table 3 gives the chromosome data available on both cell lines These studies were performed very early after initial culture as well as after serial passages in vitro. Both cell lines showed marked abnormalities with markers which can be recognized in the majority of these cells. Since individual tumors from the same tissue differed in chromosome numbers and morphology, identification of the cell line cells with tumor does not seem possible by the classic chromosome techniques.

Table 3. Chromosome data, indicating relative frequencies and degree of structural changes (From FOGH and TREMPE, 1975)

Cell Line	Modal Range	Abnormalities	Markers
SK-BR-3	Hypertriploid to hypotetraploid	+++	Large submetacentric
SK-OV-1	Hypodiploid to hypertetraploid	+++	Double ring

Table 5 List of permanent human breast tumor cell lines

Name	Originator	Year of Establishment	Solid tumor (ST) or effusion (E)	Diagnosis confirmed
BT-20	LASFARGUES, E	1958	ST	+
A1Ab	REED, M and GEY, G	1958	ST	+
SK-BR-1 III	BETH, E. and OLD, L J	1967	E	+
SK-BR-2 III	BETH, E. and OLD, L J	1967	E	+
SK-BR-3	TREMPE, G L and OLD, L J.	1970	E	+
SK-OV-1	TREMPE G L and OLD, L J.	1970	E	+
G-11	PLATA, E	1971	ST	+
SH-2	SEMAN, G.	1971	F	+
SH-3	" "	1972	E	+
HBT-3	BASSIN, R	1972		+
734-B	BREMAN, M.	1972		+
MDA-MB-157	CAILLEAU, R.	1972	E	+
MDA-MB-175-I	" "	1973	E	
MDA-MB-175-IV	" "	1973	E	
MDA-MB-175-VI	" "	1973	E	
MDA-MB-134-VI	" "	1973	E	
MDA-MB-231	" "	1973	E	+
SW-527	LEIBOVITZ, A.	1973		+
SW-613	" "	1973	ST	+
HBT-39	PLATA, E	1973	ST	+
MDA-MB-253	CAILLEAU,R	1974	E	
MDA-MB-309-I	" "	1974	E	
MDA-MB-330	" "	1974	E	
MDA-MB-331-II	" "	1974	E	
MDA-MB-331-IV		1975	E	
BT-410	LASFARGUES, E	1975		

were shown mainly in the cytoplasm and the nucleus of these cells, but most of these antigens were related to blood group substances and histocompatibility antigens.

Another type of reaction was found in the minority of tumor cells of SK-OV-1. This reaction could be initiated with the serum of patients with breast cancer and leukemia KIESTLING (1972) has searched for RNA-virus footprint in those cells without any success.

Table 5 summarizes the permanent human breast tumor cell lines available for biologic research. There have been other reports of human

breast cancer cell lines established permanently but unfortunately many of those lines are no longer available due mainly to contamination with other cell lines or with microorganisms Because of the increasing emphasis on experimental human cancer cell research and because the ultimate conquest of human cancer obviously has to be accomplished from work with the human system this type of material should be of incomparable value in further efforts in cancer research

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Chapter 6
Breast Tumor Modeling for Prognosis and Treatment*

D P GRISWOLD, JR and T H CORBETT

Cancer of the breast in women is almost unique among cancer in that it has its origin in a tissue that is not vital to life, a tissue that requires for growth and maintenance endogenous hormones, a tissue that is readily accessible to surgical removal. This suggests that the greatest portion of the tumor cell population may be surgically removed or hormonally reduced with little toxic effect on the patient and that reinduction of the disease is potentially avoidable. Such characteristics would seem to favor successful treatment and might be taken to infer that cancer of the breast would be more amenable to curative treatment than some other cancers of man.

Yet while improved therapeutic results have been observed in at least 10 other cancers during the last two decades (ZUBROD, 1972), statistical data have shown little increase in the cure rate of cancer of the breast (AXTELL and MYERS, 1975). Projections of future mortality rates, however, are probably overly liberal in that they do not take into account improved diagnostic techniques and improvements in every form of therapy. Undoubtedly, the recent report by FISHER et al. (1975) on their surgery-L-PAM trial, along with two reports on osteogenic sarcoma (CORTES et al., 1974; JAFFE et al., 1974), has now validated the concept of surgical adjuvant chemotherapy. This and other forms of combination modality therapy will very likely lead to declining mortality rates in future years. But the manner in which these various modalities and agents are combined and put to use for most efficacious results may most rapidly be determined from studies of model tumor systems. Already a number of concepts have been thus developed and appear applicable to the disease in man.

I would like to review some of the concepts and principles developed from studies of animal tumors - concepts that are relevant to the planning of therapeutic regimens and which, hopefully, will find application in the human disease. Then I will describe, with a few examples from murine tumor studies, how these concepts may apply to the selection of treatment modality, agent, and regimen in attempts to improve therapeutic results, particularly cure-rate.

The graphic illustration in Figure 1 is an oversimplification of tumor volume growth, regression, and recurrence with accompanying cell population changes. It is a composite of many views.

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* Abbreviations:

L-PAM	NSC-8806, alanine, 3-(p-(bis(2-chloroethyl)amino)phenyl)-, monohydrochloride, L-
MeCCNU	NSC-95441; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-
BCNU	NSC-409962; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

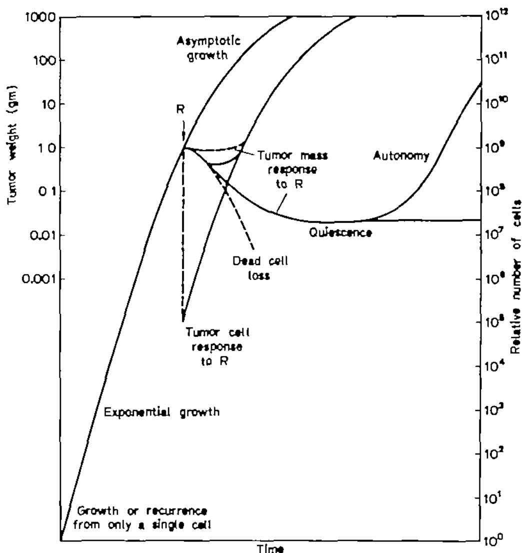


Fig 1 Graphic illustration of behavior of tumor mass and tumor cell populations during growth regression and recurrence

We know for example that growth of a number of transplantable tumors may be initiated from a single cell suggesting that total tumor cell removal or kill is necessary to effect cure SCHABEL (1975) has recently listed 15 such tumors initiated from one cell each We know too from cell titration experiments that volume growth of several transplantable solid tumors is exponential throughout much of its early history thereafter becoming asymptomatic (GRISWOLD in press) probably because of a declining growth fraction increasing cell loss or both Following successful therapy tumor volume may decrease or remain static but the nadir of regression is not an adequate indicator of the cell kill obtained from cytotoxic agent treatment (WILCOX et al 1965) Both tumor volume growth and regression are complex since they involve cell cycle time growth fraction and cell loss as well as stromal changes (BASERGA 1965;MENDELSOHN 1960; STEEL 1967) Cell death rapidly follows irradiation or cytotoxic agent exposure and for a time afterwards there is a negative correlation between tumor volume measurements and surviving viable cells detected by assay (GRISWOLD et al 1969) Associated with tumor cell repopulation there has been demonstrated an increased thymidine index and by inference an increased growth fraction since increased sensitivity to antimetabolites was

observed (LASTER et al., 1969; GRISWOLD et al., 1970). It must be pointed out, however, that those results may have been unique to a particular class of tumor and circumstances. Regardless of this fact, it is evident that a second dose or course of cytotoxic treatment should follow the first as soon as host tolerance allows.

In addition, mass regression of those tumors that are hormone dependent may be induced by the administration of exogenous steroids or by hormone-ablative procedures. The rate of cell death under those circumstances has not been precisely measured, but it is speculated that numbers of viable cells may more closely approximate changes in tumor volume. Regressed tumors may recur following cessation of treatment with exogenous steroids, adaptive changes in endogenous hormone levels, or because of autonomy. These characteristics appear to obtain both in hormone-dependent breast tumors of rat and man (GRISWOLD and GREEN, 1970; McGUIRE, 1974)

It is evident, then that control of those tumor cells that retain proliferative integrity is the key to suppression of tumor growth and increased longevity of the host. But since it is unlikely that tumor growth inhibition may be maintained indefinitely, "cure" is the other alternative and that is dependent on reduction of the tumor cell population to small numbers and perhaps to less than one viable cell.

But what of those cells, in the course of treatment, that appear to be dead or doomed to die? Laboratory evidence suggests that they should not be ignored. RÉVÉSZ (1956) and subsequently a number of other investigators, De WYS (1972); De WYS and KNIGHT (1969); DECKERS et al., (1971) showed that radiation-inactivated tumor cells, when implanted with viable tumor cells into mice, would support the growth of those tumor cells that were otherwise insufficient in number to establish tumors. We have more recently shown that the same phenomenon will result from the use of drug inactivated tumor cells, i.e., cells that have been exposed in vitro or in vivo to a drug that caused proliferative death (DYKES, personal communication) (Table 1).

Table 1. Tumor incidence of B16 melanoma following s c implantation of counted cells with MeCCNU- or Co^{60}-killed tumor cells No tumor growth resulted from implantation of feeder cells alone

No. of Viable cells	MeCCNU-FC[a]	Co^{60}-FC[a]	No FC[a]	
10^7	39/39 (100)	40/40 (100)	37/39 (95)	
10^6	37/38 (97)	39/40 (98)	12/40 (30)	TD_{50}[b]
10^5	39/40 (98)	34/34 (100)	1/40 (3)	
10^4	36/40 (90)	31/39 (80)	0/40 (0)	
10^3	31/40 (78)	24/40 (60)	0/40 (0)	
10^2	12/40 (30)	15/40 (38)	0/40 (0)	
10^1	3/40 (8)	2/40 (5)	0/40 (0)	

[a]Feeder cells were prepared by exposure in vitro to 10,000r Co^{60} or in vivo to 160 mg/kg MeCCNU, approximately 10^8 feeder cells were added to each "viable" cell implant

[b]TD_{50} is that number which when implanted results in tumor growth in 50% of the mice

Shown here are tumor incidences following s c implantation of graded numbers of B16 melanoma cells alone or in the presence of tumor cells previously killed by gamma irradiation in vitro or by MeCCNU in vivo The four- to fivefold differences in tumor take-rates clearly indicate the effect that cells which have lost proliferative integrity may have on their viable cohorts

Although the exact mechanism is not known by which reproductively dead cells may serve as so-called feeders we may assume that so long as they retain metabolic integrity and until lost from the tumor mass they may encourage the maintenance of life and subsequent reproduction of cells that might otherwise be doomed to die This phenomenon has far-ranging implications

In the hormone dependent tumor another problem may exist The concept that hormone dependent tumors may at some later time become hormone independent may be too conclusive suggesting that hormone-dependence or independence is an all or none condition It largely overlooks the work some years ago of YOUNG and COWAN (1963) and of DANIEL and PRICHARD (1964) whose histologic studies of regressing hormone-dependent rat mammary tumors showed evidence of growth in localized areas within some of the regressing tumors Believing hormone-dependent tumor growth following ablation to be a function of lessened but continued estrogen output it became common to follow ovariectomy with adrenalectomy and then hypophysectomy to further diminish estrogen sources (STOLL 1969)

YOUNG and COWAN further showed that hormone dependent rat tumors that had regressed following ovariectomy could be stimulated to grow by appropriate steroid treatment Also using the 7 12-dimethylbenz(a)anthracene(DMBA)-induced rat mammary tumor as a model (Fig 2) we have shown that the growth of tumors regressed below a palpable level by ovariectomy may be initiated by treatment with progesterone and estradiol at any time Since the time of regrowth was directly related to the time of initiation of treatment and that time difference was approximately constant regardless of when treatment was started we concluded that a small but relatively stable population of hormone-dependent cells persisted in those regressed quiescent tumors Furthermore evidence of an enhanced rate of growth during steroid treatment of so-called autonomous tumors i e treatment of those that had already begun to recur following ovariectomy-induced regression led us to believe that both hormone-dependent and -independent cell populations might exist within the same tumor mass Tumor regrowth following hormone-induced regression might then simply reflect a changing ratio of those two populations suggesting that treatment from the outset should be directed at both populations Of course because of the work of JENSEN et al (1966); McGUIRE et al (1975); and HORWITZ et al (1975) on the detection of hormone receptors it is now possible to identify some if not all patients who may benefit from hormonal alterative procedures

Tumor volume increase regression and recurrence are not complete mysteries but are dependent on changing population sizes Host survival time as well as tumor size are measurable and predictable on the basis of direct and indirect measurements

It has been shown from cell titration experiments with a number and variety of animal tumors that a direct relationship exists between the size of the tumor cell implant and the latent period for tumor development as well as the time of host death (GRISWOLD in press; SKIPPER 1970) It has further been shown that the extent of meta-

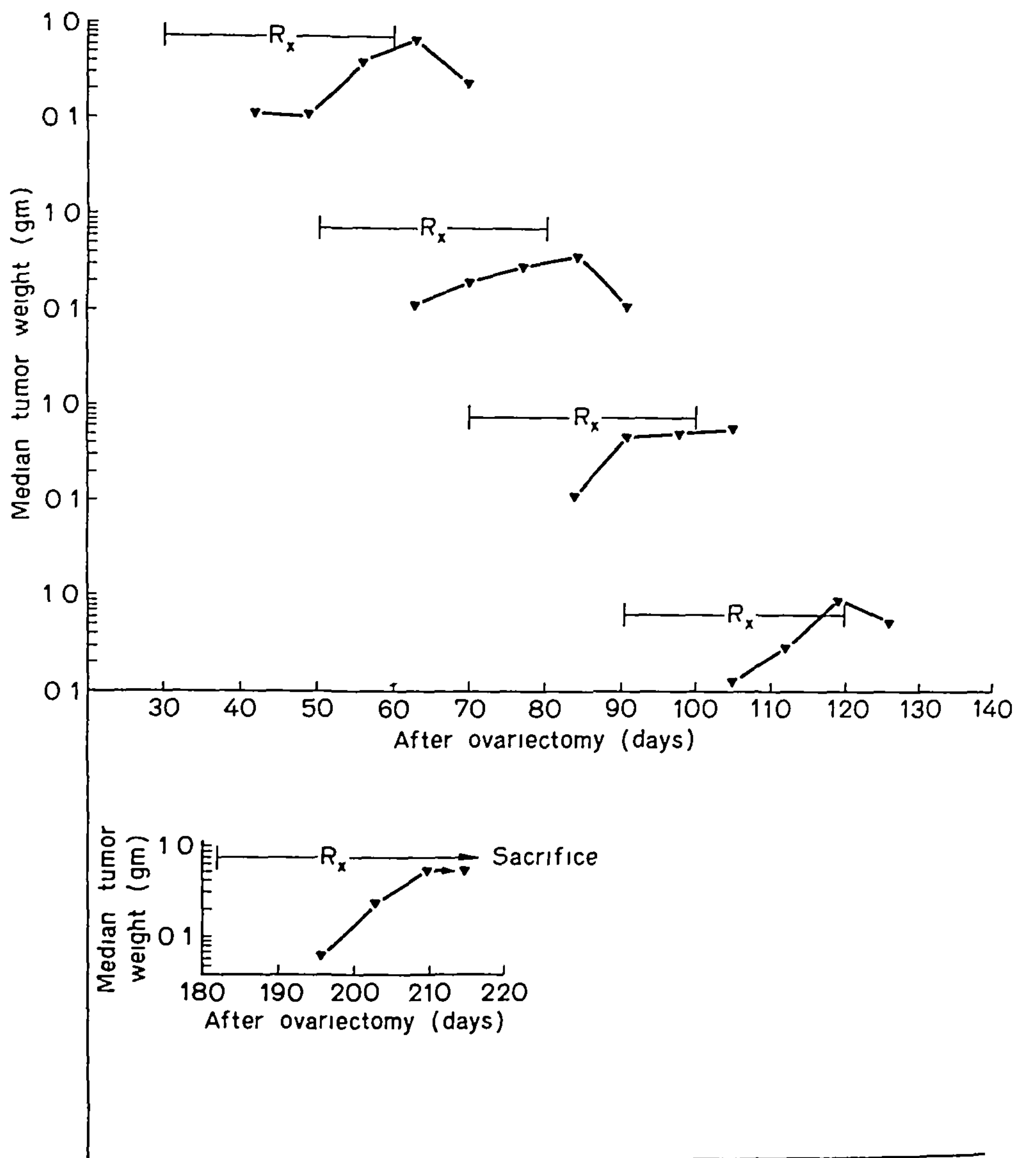


Fig 2 Growth stimulation of 7,12-dimethylbenz(a)anthracene-induced mammary tumors in female, Sprague-Dawley rats following ovariectomy-induced regression Rats whose tumors had regressed below a palpable level and remained so were treated with progesterone, 8 0 mg/kg/day and estradiol,17-ß, 2 0 µg/kg/day for periods indicated Tumor weights were calculated from caliper measurements

stasis from a subcutaneous (s.c.) tumor implant is directly related to time following primary tumor implantation (GRISWOLD, 1972). Experimental results also indicate that the fraction of animals cured of the: tumors is inversely related to the stage of the primary tumor at the time of initiation of treatment. This appears to hold true for both surgical treatment and chemotherapy.

When mammary adenocarcinomas were implanted s.c. into C3H mice, then su gically removed when those tumors had reached 100-200 mg, 250-300 mg, c 500-700 mg, the cure rates were, respectively, 57%, 29%, and 2 0% (Fig. 3). The remaining animals died of metastatic disease. Similarly, when mice were implanted s.c. with 10^7, 10^5, or 10^3 C3H mammary tumor cells, then treated with a single dose of 40 mg/kg of MeCCNU two days later (Fig.4) 48% of all treated mice, i.e., the three pooled groups, survived 120 days tumor-free while only 13% of all untreated mice survived. But con-

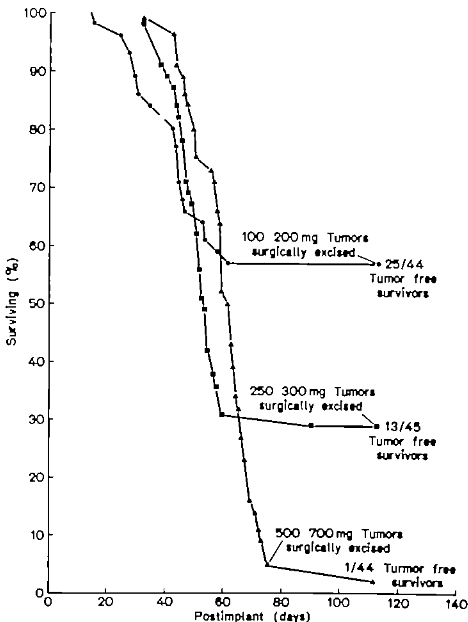


Fig 3 Survival of mice bearing s c -C3H mammary adenocarcinoma following surgical excision of the s c -tumor at various stages of growth (reproduced with permission of Cancer Chemotherapy Reports)

tributing most heavily to that 48% overall rate among the pooled treated mice were those bearing only 10^3 cells with 90% long-term tumor-free survivors and those with 10^5 cells and 56% survivors There were no survivors in the 10^7 cell-treated group Thus it would appear from these animal tumor studies that both chemotherapeutic cure and surgical cure are dependent on tumor stage

If then one accepts the premise that tumor growth regression and recurrence along with changes in tumor cell population numbers may be reasonably well defined and that tumor size is an important factor in prognosis as well as diagnosis then the current state of art may be graphically visualized if only crudely as follows (Fig 5) It must be emphasized that this description does not take into account the wide ranges often observed in tumor growth characteristics nor ranges

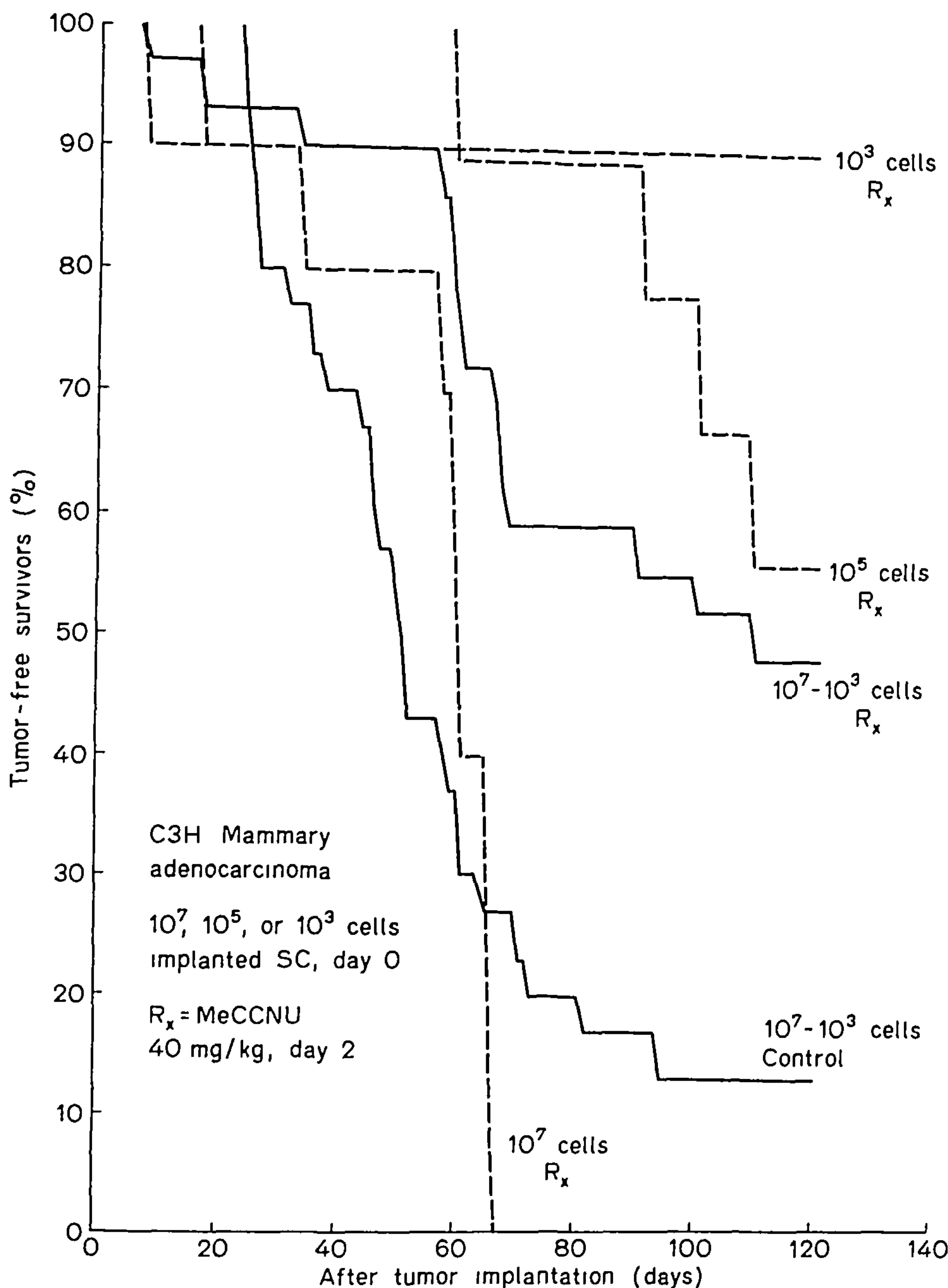


Fig 4 Influence of tumor stage on chemotherapeutic results. Mice were implanted s c with 10^7, 10^5, or 10^3 C3H mammary adenocarcinoma cells with lethally irradiated Co^{60} feeder cells prior to drug treatment ------ individual groups ——— pooled groups

in diagnostic and therapeutic capabilities Furthermore, it is not necessarily true that the growth rates of the primary and metastatic tumor are identical, as shown here. Nevertheless, we may assume that the size of any metastatic tumor will lag behind that of the primary, at least for awhile. Such a situation offers promise of a favorable prognosis and successful treatment if the primary is resectable and if the number of cells in any metastatic site is within the range of cure. Unfortunately, I am told that the usual size of the primary breast cancer at diagnosis is about 2 5 cm or roughly 10g. Thus, in this illustration, the number of metastatic cells (more than 10^7) would be beyond the reach of currently available systemic therapy. The diagnostic potential, however, is much better even today, than 2.5 cm I

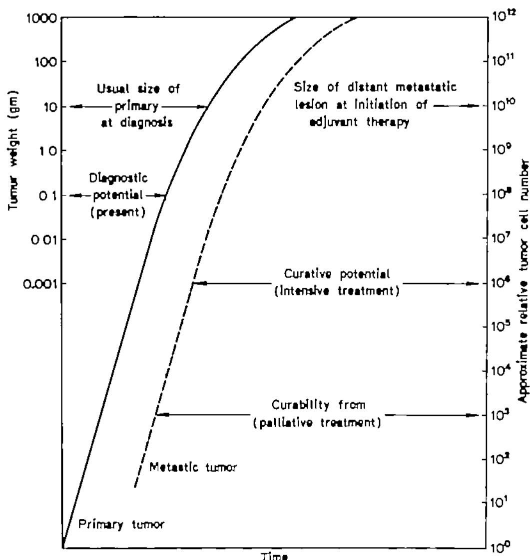


Fig 5 Hypothetical illustration of relationship between primary and metastatic tumor foci in regard to diagnostic and therapeutic potential

have arbitrarily shown it here as 1 0 g or about 1 0-1 5 cm This difference on the average puts the metastatic tumor cell burden within the range of cure if systemic therapy were intensive More conservative forms of treatment although increasing survival time and duration of remission would probably not be expected to be curative

We recognize of course that while this may appear to be a neat concept for the treatment of animal tumors it presents a serious ethical problem in man namely that of treating patients without detectable signs of disease We should hope however that both the diagnostic potential and therapeutic potential will increase to the point that the horizontal lines shown here will overlap that the size of the tumor that is diagnosable will equate with that size that is curable

Thinking now only of chemotherapy how may it be made more effective? Aside from the development or isolation of more effective single agents a number of problems may be visualized as obstacles to more successful chemotherapeutic results from currently available agents Among these

are selection of the proper sequences, ratios, dosages, and treatment schedules, and most importantly, selection of an effective agent or combination of agents for a given tumor.

In our laboratories we have developed more than 20 transplantable mammary tumor lines, each derived from a single spontaneous mammary adenocarcinoma in a C3H mouse. Four of them are listed here (Table 2). They vary in degree of differentiation and in certain biological characteristics, but more importantly, each has a distinct response pattern to chemotherapy. For example, while tumor line 04/A is markedly sensitive to 6/7 agents listed, line 44 is markedly sensitive to only 1/7. With the exception of cyclophosphamide and methotrexate, sensitivity of the four tumor lines to the remaining five agents is remarkably variable, ranging from excellent to none.

Not only does chemotherapeutic sensitivity vary from one tumor line to another, it may vary from tumor to tumor within a single line (Table 3). When first generation transplants of the spontaneous Martin-Fugmann (MARTIN et al., 1970) mammary tumor were used for evaluation of L-sarcolysin and adriamycin, we found that the percentage of tumors responding to treatment with the two agents combined was additive, as compared to the percent age after treatment with each of the single agents. One explanation suggests that, among those treated with the two-drug combination, one subgroup responded to one agent while another subgroup responded to the second agent - in that sense, true additivity, not potentiation. This evidence of heterogeneity of response poses a serious problem to the clinician, particularly when he cannot, for a time, measure the effectiveness of his choice of treatment

It has been common clinical practice to consider as active those agents that had individually been shown to be useful in the treatment of very advanced disease, often in patients who had previously been treated by other methods. Failure of a particular agent to demonstrate antitumor activity under those conditions then relegated that agent to the category of inactives. But perhaps that criterion is too stringent. Perhaps potentially useful agents in less advanced disease are being overlooked.

Table 2 Arbitrary response rating of four transplantable mammary tumor lines[a] to selected anticancer agents For agent evaluation, tumor fragments were implanted s c into mice which were then treated with LD_{10} doses and fractions thereof, using a variety of treatment schedules These results represent our total experience for the agents and tumors indicated

	Tumor line			
	04/A	15/C	16/C	44
Cyclophosphamide	+++	+++	+++	+++
L-Sarcolysin	+++	-	++	+
Adriamycin	+++	++	+++	+
5-FU	+++	+++	++	-
Vincristine	+++	-	++	-
Prednisone	+++	-	+	-
Methotrexate	-	-	-	-

[a] Each line derived from a separate spontaneous breast tumor of C3H mice

Table 3 Response to chemotherapy of first generation transplants of CD_8F_1 mammary adenocarcinomas

Treatment[a]	Tox control mortality	% Ils	% Responders	Response duration
L-Sarcolysin 8 0 mg/kg/dose	1/10	33	30	36 days
Adriamycin 6 0 mg/kg/dose	0/10	70	40	51 days
L-Sarcolysin 5 4 mg/kg/dose + Adriamycin 4 0 mg/kg/dose	1/10	45	70	46 days
L-Sarcolysin 5 4 mg/kg/dose + Adriamycin 2 6 mg/kg/dose	0/10	64	60	49 days

[a] Tumor fragments were implanted s c All treatment was Q7DX5 beginning 2 days after tumor implantation (1450 F_1)

The phenomenon, that certain agents are ineffective in advanced disease but effective against small tumor cell populations has been noted in animal tumor studies As an example (Fig 6) we treated mice bearing large 1 0 g or larger primary mammary tumors with a single dose of the nitrosourea BCNU The dose was approximately an LD_{10} and was given about 6 days before the first observed death in the untreated control By any interpretation that treatment would be judged ineffective against this metastatic tumor since all the mice died at about the same time as did those in the untreated control However when the primary tumors were removed and mice were treated with the same dose of the same agent 40% survived tumor-free as compared to none in the surgery control and those that died had an extension in survival time

Unquestionably the selection of chemotherapeutic agents for use in man is difficult But until such time that the drug sensitivity of individual patients cancers can be determined agent selection on the basis of probability of response remains the alternative

A number of concepts as well as problems have now been noted or inferred, and it might be well to show now how some have been applied to the treatment of animal tumors thinking of success or failure primarily in the sense of cure

In our experience with the DMBA induced mammary adenocarcinoma in the rat we found those tumors to be relatively insensitive to a variety of chemotherapeutic agents (GRISWOLD et at 1966) Although not metastatic multiple tumors appear in each rat after induction Thus we found it useful in attempts to treat early disease to surgically remove the first that became detectable then direct systemic therapy at those tumors not yet palpable (Fig 7) In this experiment where we measured the percent of tumor-free survivors over a period of time after surgical excision of the first tumor to appear treatment with cyclophosphamide alone was without effect Treatment of those hormone-dependent tumors with the androgen 2-α-methyl-dihydrotestosterone propionate delayed their appearance but after cessation of treatment tumors appeared in all rats so treated In contrast more than 40% of

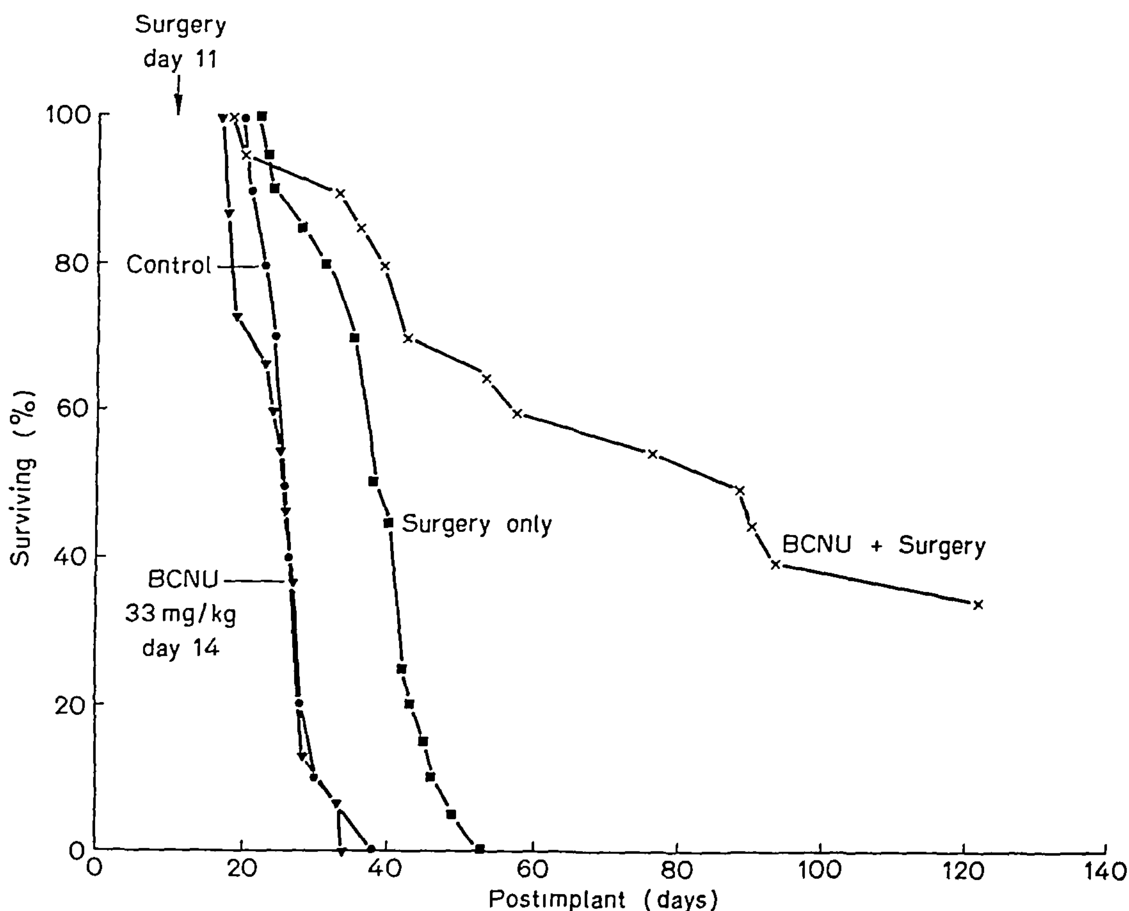


Fig 6. Effectiveness of BCNU against advanced C3H mammary adenocarcinoma with or without surgical removal of the primary, s.c tumor Median primary tumor weight at surgery was 1.3 g

rats treated with the combination of cyclophosphamide and the androgen failed to develop new tumors as late as nine months after surgery One explanation for this result could be that the bulk of the tumor cell population was reduced by the androgen while cyclophosphamide killed the few surviving cells.

In any discussion of chemotherapy, the selection of dosage and schedule must be prime considerations. I have previously alluded to the distinction between palliative and intensive treatment. But I would like to go a step beyond that and suggest that even treatment to toxicity may be inadvertently palliative (Fig. 8). This idealization of tumor cell kill following treatment was prepared from a large body of pooled data collected from several experiments with the s.c.-implanted B16 melanoma. In this study, mice were treated with one of three schedules: once only, four times at four-day intervals, or once daily for nine days. All groups were treated with the same agent, cyclophosphamide, using dosages that were approximately equitoxic. Although the duration of tumor remission (indicated as T-C in days) as well as the increased host life span of mice in the three groups suggests comparable antitumor effect, it is evident that the numbers of cells killed (measured in $\log_{10}$ units) are quite different. In this particular case, the single dose was more than twofold better than either of the other two treatment schedules. Certainly, I would not advocate this schedule in every situation, for the selection of drug dosage and schedule is dependent on characteristics of drug, tumor, and normal tissues Never-

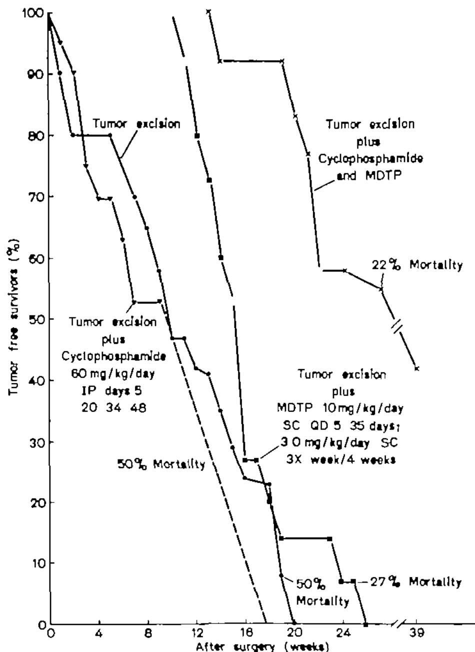


Fig 7 Response of 7 12-dimethylbenz(a)anthracene-induced mammary tumors in rats to cyclophosphamide 2-a-methyldihydrotestosterone propionate or combination See text for details

theless the effect that treatment schedule may have on the success of treatment must be noted In this instance had there been fewer cells to be killed say 10^4–10^5 the effect of treatment schedule selection would be obvious in that only one of these schedules would be predicted to be curative

As perhaps a better example of the effect of intensity of treatment (Fig 9) a simple comparison of curative results from high-dose or low-dose chemotherapy following surgery may be pertinent particularly since low-dose chemotherapy has recently been advocated as an adjuvant to surgery of the breast in women (CREECH et al 1975) In this experiment C3H mammary tumors were implanted s c then surgically

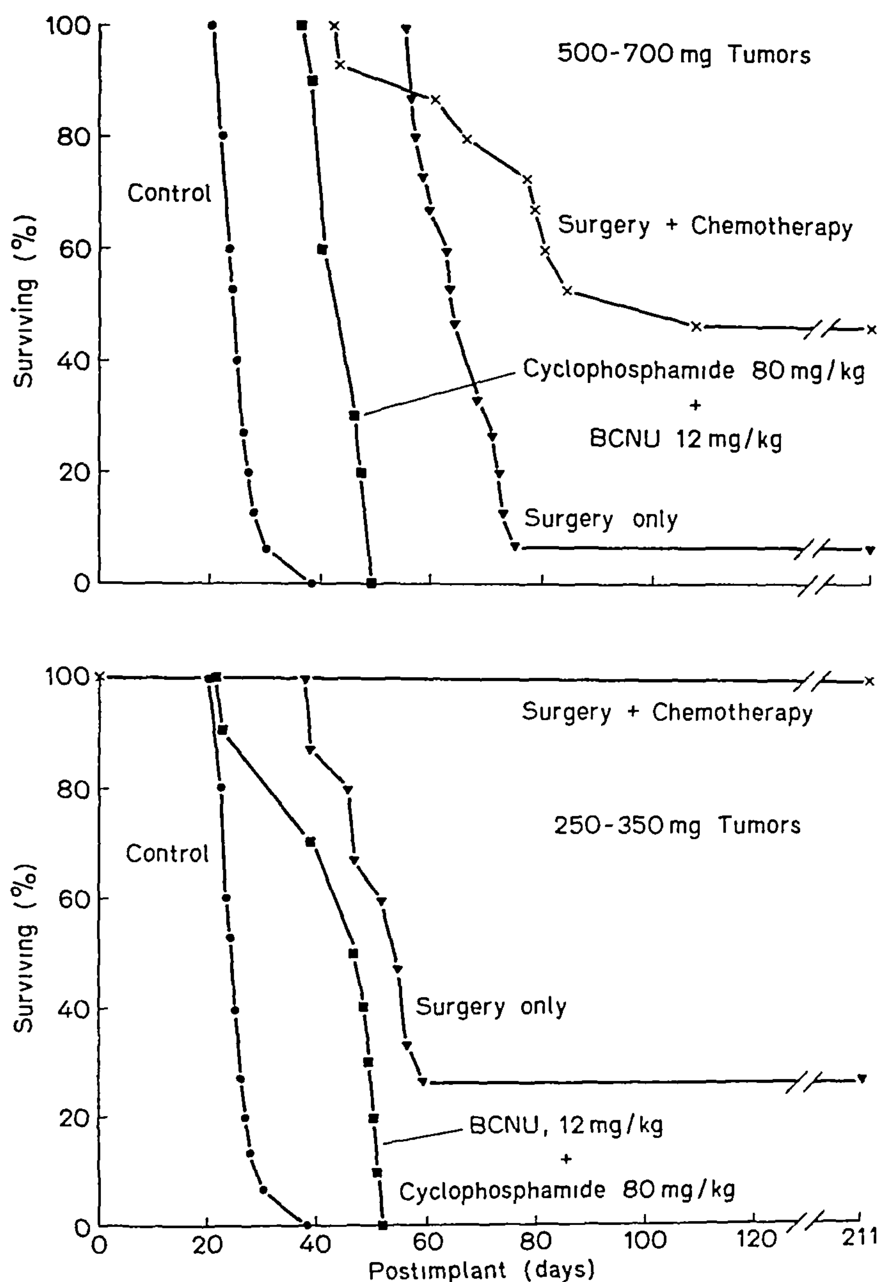


Fig 10 Tumor stage effect on surgery-chemotherapy results. C3H mammary adenocarcinoma Tumor fragments were implanted s c by trocar Those primary tumors were surgically removed at a time when median weight was about 300 mg or about 600 mg Chemotherapy was given 4 days later in either case

component should not be ignored. Those proliferatively dead but metabolically active cells may encourage the survival of otherwise doomed-to-die cells, thus interfering with subsequent cell killing attempts and/or allowing for tumor growth lags that may misleadingly suggest more favorable treatment results than actually occurred.

4. So-called hormone dependent tumors may be mixed populations requiring simultaneo tment t the autonomous fraction as well as the horm nt

5 Therapy from the moment of design is either curative or palliative including particularly chemotherapy There is no question at this point that agents with greater selective toxicity are needed But the usefulness of currently available agents may not yet have been fully exploited for success or failure is dependent to a great extent on the selection of agent dosage and schedule of administration

In conclusion I would re-emphasize that the data presented are from animal studies only Several models were used to derive concepts that hopefully may be translatable to man The practical application of these concepts however must await trial in man Until such time as precise knowledge of the response of human breast cancer to treatment is available most of what is done will of necessity be based on probability In that regard it is then perhaps appropriate to clinical therapy design and to this conference to quote Descartes French mathematician and philosopher who in his Discourse on Method wrote It is a truth very certain that when it is not in our power to determine what is true we ought to follow what is most probable

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Chapter 7
Estrogen Receptors and Hormone Dependency in Human Breast Cancer

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INTRODUCTION

Endocrine ablative and additive hormone therapies have been used widely in the treatment of advanced breast cancer since Sir George Beatson's report in 1896 describing remissions in response to bilateral oophorectomy (BEATSON 1896) Either removal of endocrine producing organs or administration of pharmacologic quantities of hormones results in objective remissions in 25-40% of patients with advanced breast disease (KENNEDY 1974) Recently, the ability to predict with confidence the breast cancer patient most likely to respond to endocrine manipulation was demonstrated using estrogen receptor analyses (e g JENSEN et al 1971; WITTLIFF 1974; McGUIRE et al 1975)

INTERACTION OF ESTROGEN WITH TARGET CELLS

The proposed sequence of events which follow the interaction of estrogen with a breast cell is shown in Figure 1 The scheme of events outlined evolved from the original two-step mechanism suggested independently by GORSKI et al (1968) and by JENSEN et al (1968) for the association of estradiol-17β with uterine cells As lipids the steroid hormones apparently enter the cell by passive diffusion and combine with a cytoplasmic form of the estrogen binding protein designated

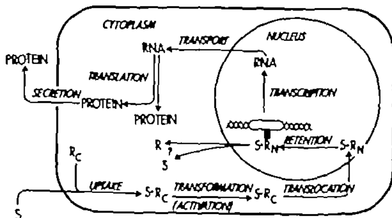


Fig 1 Proposed steps in interaction of steroid hormone with breast cell Cytoplasmic form of receptor is designated as R_C nuclear form as R_N and steroid hormone as S

R_C. Formation of the estrogen-receptor complex is characterized by high affinity and ligand specificity. Although R_C has been reported present in cytosol, i.e., cytoplasm devoid of subcellular particles, its exact location in the target cell is unknown currently. It may be bound loosely to the inner surface of the cytoplasmic membrane (or to microsomes) and released during homogenization (LITTLE, et al., 1973; WITTLIFF et al., 1976). Prior to translocation, the estrogen receptor complex apparently must undergo an activation step which requires association of the steroid hormone (JENSEN et al., 1971b). Once inside the nucleus, the steroid-receptor complex combines with the chromatin and stimulates RNA synthesis and subsequent formation of certain breast cell proteins (Figure 1). "Steroid binding proteins" or "steroid receptors" are required elements of the mechanism of action for each of the classes of steroid hormones on target cells (JENSEN et al., 1974; HILF and WITTLIFF, 1974, 1975; BULLER and O'MALLEY, 1976). Normal breast tissue contains specific binding sites for estrogens, progestogens, glucocorticoids, and androgens (WITTLIFF, 1975).

A molecular difference between responsive and unresponsive breast tumors was implied originally by the findings of FOLCA et al. (1961) that certain patients exhibiting remissions to adrenalectomy had carcinomas which concentrated large amounts of labeled hexestrol, a synthetic estrogen. Later JENSEN et al. (1971a) suggested more specifically that the presence of estrogen receptors in breast carcinomas was predictive of a patient's response to endocrine therapy. Investigations from our laboratory and those of others have now established that the estrogen receptor provides a molecular index of hormone dependency of a breast tumor (cf. WITTLIFF, 1974, 1975; McGUIRE et al., 1975).

COLLECTION AND HANDLING OF BREAST TUMOR SPECIMENS

If estrogen receptor analyses are to be used in the selection of endocrine therapy for breast cancer patients, a number of practical considerations are warranted. First, it is important that the tissue be frozen in liquid nitrogen or on dry ice as soon after excision as possible. This precaution is necessary principally because of the lability of estrogen receptor in breast tissues (GARDNER and WITTLIFF, 1973; WITTLIFF and SAVLOV, 1975). Pathologists in our hospitals often prepare tumor biopsies at the time of frozen section diagnosis using the freezing microtome. If the tissue must be transported to the biochemical laboratory prior to freezing, it is also important to pack it in ice, but not in direct contact.

For convenience of freezing and storage of breast tissues we have utilized polyethylene vials with snap caps of the type used by electron microscopists. These resist breakage due to freezing in liquid nitrogen and hold approximately 0.5 g of tissue. The tissues must be stored at -70 - -86° C in an ultra-low freezer such as those sold by Kelvinator or Revco Additionally, a number of tissues may be stored and then shipped on dry ice to a central laboratory for estrogen receptor analyses.

At the time of extraction of estrogen receptors, one may take the frozen tissue and shatter it to a powder in a device termed an auto-pulverizer (Thermovac Industries). This instrument is cooled in liquid nitrogen to keep the breast tissue frozen during pulverization. Then the powder is extracted in all-glass homogenizers using procedures reported earlier (WITTLIFF et al., 1972; WITTLIFF, 1975; WITTLIFF and SAVLOV, 1975). This method is particularly useful in analyzing bone

biopsies and fibrotic and calcified lesions Prior to estimation estrogen binding capacity a representative sample of the breast biopsy is fixed in neutral formalin stained with hematoxylin and and examined microscopically Confirmation of tumor pathology and quantitative estimation of cellular composition are performed as ported earlier (WITTLIFF et al , 1972; WITTLIFF and SAVLOV 1975)

METHODS OF ESTROGEN RECEPTOR ANALYSES

Estrogen receptors in extracts of breast tissues have been estima using a number of methods which range from administration in vivo [^{3}H]steroids to simplified procedures for estimation of binding s in vitro (cf WITTLIFF 1975) Since the concentration of recepto sites is extremely low the only manner of detection presently av able is by combination with tritium-labeled estradiol-17β of high cific radioactivity Under appropriate conditions of pH and tempe the radioactive estrogen combines with the receptor site in a tum extract forming the estrogen-receptor complex The principal pro in all binding protein assays has been to separate bound from unb steroid In early studies of hormone-binding administration in v of labeled hormones and incubation of tissues slices with labeled mones were used However neither of these methods appears to be tical for routine clinical use Two procedures which the authors utilized over the past six years are the absorption of unbound ho to dextran-coated charcoal and the sucrose-gradient centrifugatio method Analyses by both of these procedures require 300-500 mg o tissue However we have performed sucrose gradient analyses on a as 90 mg Details of these methods are contained in our earlier r (WITTLIFF et al , 1972; GARDNER and WITTLIFF 1973; SAVLOV et al WITTLIFF 1974 1975; WITTLIFF and SAVLOV 1975)

SEPARATION OF MOLECULAR FORMS

Using sucrose gradient centrifugation we have determined that th sedimentation profiles of estrogen-binding components in cytosol tions of human breast carcinomas fall into four general categori (WITTLIFF 1974): specific estrogen receptors which sediment at 8-9S, 4-5S or both 8-9S and 4-5S as shown by the representativ files in Figure 2 or undetectable (not illustrated) Unlabeled ethylstilbestrol was used as a competitive inhibitor of binding sure that only specific association was measured The proportion two forms of estrogen receptors varied considerably among tumors (WITTLIFF and SAVLOV 1975) The 8S profile (Fig 2A) is typical that seen in normal breast tissue such as the lactating mammary of the rat (GARDNER and WITTLIFF, 1973) The profiles shown in F 2 illustrate that both the 8-9S and 4-5S species did associate w [^{3}H]estradiol-17β in a specific fashion since binding was inhibi by unlabeled diethylstilbestrol By competition analyses with se unlabeled steroid hormones additional evidence was found that e the molecular forms of these components bound [^{3}H]estradiol-17β cifically (Table 1) It should be noted from the data in Table 1 approximately 50% of the binding under the 4-5S peaks in the tis examined was *not* inhibited by unlabeled estrogens We attribute t residual binding capacity to association of [^{3}H]estradiol-17β wi sites such as those on serum albumin which bind estrogens with l affinity and high capacity The quantity of nonspecific binding

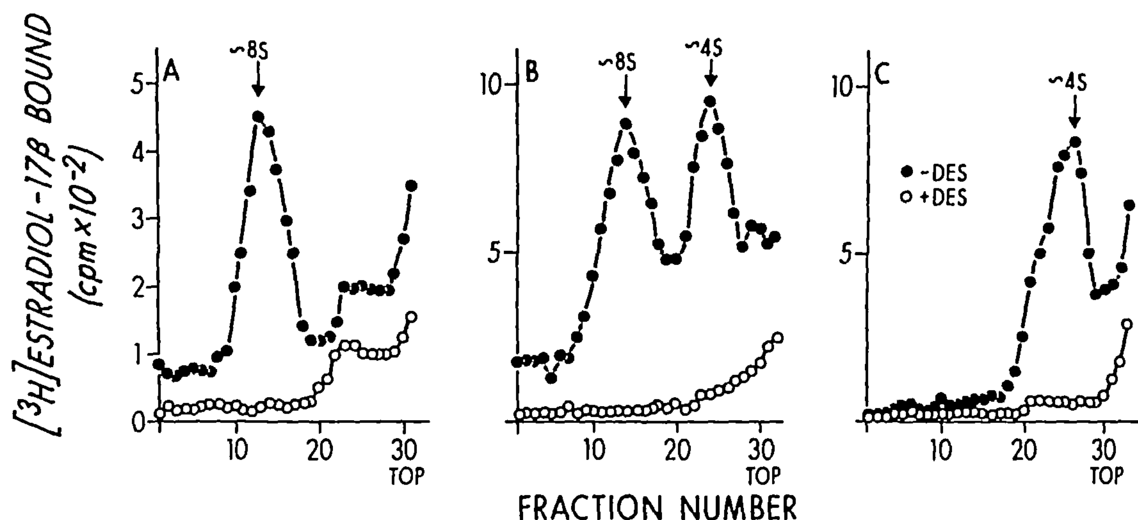


Fig 2 Estrogen receptors in infiltrating ductal carcinoma of female breast Cytosols were reacted with [^3H]estradiol-17β (4-6nM) for 5 h at 3°C in the presence (open circles) and absence (closed circles) of 250-fold excess unlabeled diethylstilbestrol (DES). Each reaction was treated with a pellet of dextran-coated charcoal prior to separation by sucrose gradient centrifugation (Taken from WITTLIFF et al , 1976)

the 4-5S region varied greatly among specimens (e.g , Figs. 2 and 4) Neither androgens, progestogens, nor glucocorticoids were competitive for estrogen-binding sites indicating further the ligand specificity of both the 8-9S and 4-5S species of estrogen receptors in human breast tumors

Table 1 Specificity of ligand binding by estrogen receptors in human breast cancer

Competitive substance[a]	[^3H]Estradiol-17β bound (%)[b]	
	8-9S Species	4-5S Species
None	100	100
Estradiol-17β	3±1	53±14
Estrone	4±1	54±13
Diethylstilbestrol	3±1	46±10
Dihydrotestosterone	85±3	81±3
Progesterone	82±10	97±1
R5020	95[c]	91[c]
Hydrocortisone	90±6	98±1
Corticosterone	98[c]	82[c]
Triamcinolone acetonide	99±1	94±3

[a] Concentration was 250- to 500-fold greater than that of [^3H]estradiol-17β

[b] Values presented represent mean ±SEM, 5 tumor specimens

[c] Single sample

LIGAND AFFINITY MEASUREMENTS

In a recent report the question of the ligand affinity of these molecular forms of the estrogen receptor was raised (WITTLIFF and SAVLOV 1975) To accomplish this we have examined several breast carcinomas which were suitably large by both the sucrose gradient and dextran-coated charcoal procedures Using the same cytosol preparation from each of the three tumors whose estrogen receptor profiles are presented in Figure 2 the specific binding sites were titrated with increasing concentrations of [^{3}H]estradiol-17β (Fig 3) The titration curve for the cytosol containing predominantly 8-9S binding components is shown in Figure 3-1A The dissociation constant (K_d) of these estrogen-receptor complexes was 4 X 10^{-10} M obtained from the Scatchard plot shown in B From the Scatchard analysis of the titration curve shown in Figure 3-2 a K_d of 3 X 10^{-10} M was obtained for the cytosol containing a mixture of 8-9S and 4-5S estrogen receptors In the cytosol containing only the 4-5S estrogen receptors Scatchard analysis of specific binding data (Fig 3-3A) gave a K_d of 7 X 10^{-10} M These data indicate that although the binding components differ in their sedimentation properties on sucrose gradients of low ionic strength their affinity constants for [^{3}H]estradiol-17β were similar The values described here were com-

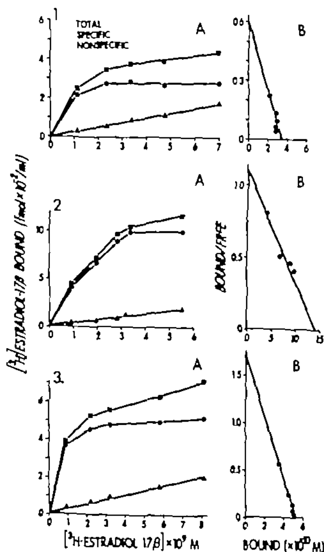


Fig 3 Titration of estrogen-binding sites in human breast carcinomas Each of the exact cytosols containing estrogen receptors shown in sucrose gradients in Figure 2 was titrated simultaneously with [^{3}H]estradiol-17β using dextran-coated charcoal method Titration curve and Scatchard plot of cytosol containing 8-9S estrogen receptors are presented in parts 1 A and B those for cytosol exhibiting both the 8-9S and 4-5S components are shown in parts 2 A and B while those for cytosol containing 4-5S species are given in parts 3 A and B (Taken from WITTLIFF et al 1976)

parable to those reported earlier (WITTLIFF et al., 1972; HÄHNEL and TWADDLE, 1973).

ESTROGEN RECEPTORS IN BREAST CARCINOMA OF THE MALE

Specific estrogen-binding components also were demonstrated in primary and metastatic breast carcinoma of the male (Fig. 4). As shown, these tissues exhibited both the 8-9S and 4-5S species of estrogen receptors using the sucrose gradient procedure. As described earlier (Fig. 2), both of these components did associate with [^{3}H]estradiol-17ß in a specific fashion as indicated by the decrease in binding in the presence of unlabeled diethylstilbestrol, a competitive inhibitor. The specific estrogen-binding capacity of the cytosol from the primary tumor was 96 fmoles/mg cytosol protein distributed equally between the two species of estrogen receptors. The breast metastases to the lymph node contained 37 fmoles/mg protein of 8-9S species and 31 fmoles/mg protein of 4-5S type. The normal breast tissue peripheral to the primary lesion did not, however, contain estrogen receptors (Fig. 4C) Examination of cytosol from several samples of gynecomastia also did not indicate the presence of estrogen-binding proteins.

Evidence that the components in cytosol of breast tumors from males bound estradiol-17ß specifically with high affinity is presented in Figure 5. Cytosol from an infiltrating ductal carcinoma of the breast was titrated with increasing concentrations of [^{3}H] estradiol-17ß in the presence and absence of unlabeled diethylstilbestrol using the dextran-coated charcoal procedure. Scatchard analysis of the specific

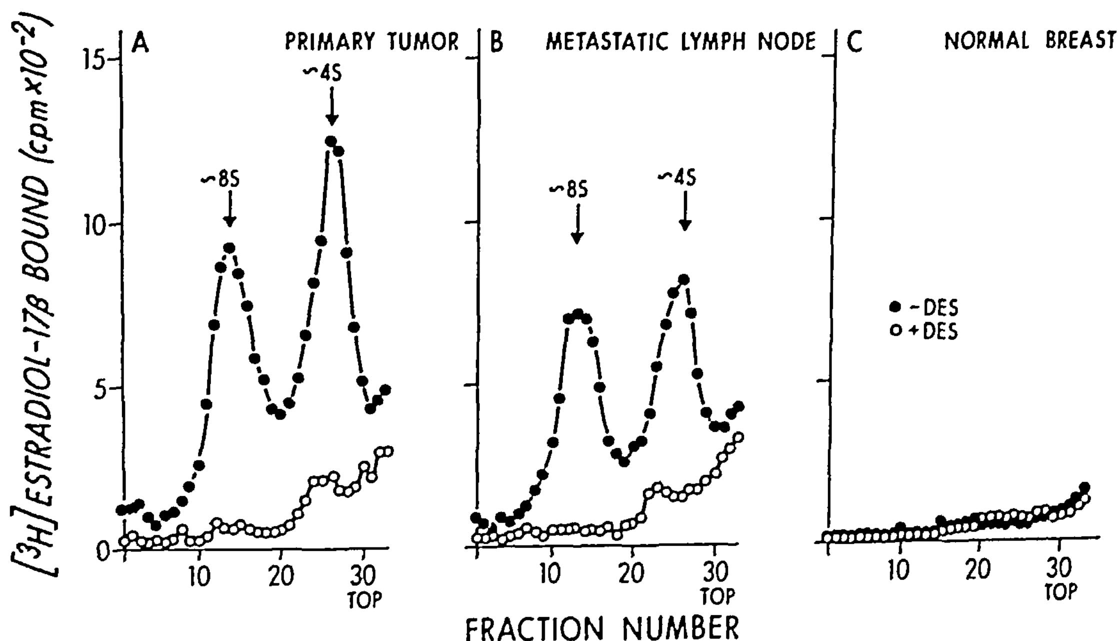


Fig 4 Estrogen receptors in tissues from a male patient with infiltrating ductal carcinoma of the breast. Cytosols were reacted with [^{3}H]estradiol-17ß(4-6nM) for 5 h at 3OC in the presence (open circles) and absence (closed circles) of 250-fold excess unlabeled diethylstilbestrol (DES). Each reaction was treated with a pellet of dextran-coated charcoal prior to separation by sucrose gradient centrifugation

binding data revealed a K_d value of 5 X 10^{-10} M; the number of binding sites for this preparation was 96 fmoles/mg cytosol protein (Fig 5B)

Earlier we described estrogen receptors in specimens of metastatic breast cancer of males (WITTLIFF 1974, 1975) The results presented in Figure 4 illustrate the observation that often the distribution of molecular forms of estrogen receptors in the primary lesion also are found in the metastases If the estrogen-binding capacity of the primary disease reflects that of the metastatic lesions one may be able to predict the endocrine responsiveness of advanced breast carcinoma prior to its presentation clinically

DISTRIBUTION OF ESTROGEN RECEPTOR SPECIES

As defined earlier (WITTLIFF et al , 1972) levels of estrogen receptors were categorized according to the quantitative estrogen-binding capacity If the estrogen-binding capacity was less than 3 fmoles (10^{-15} moles)/mg cytosol protein the tumor was designated estrogen receptor negative When the value was 3-7 fmoles of binding the content was borderline whereas a binding capacity of 7 fmoles/mg cytosol protein or greater was considered receptor positive

Table 2 summarizes the distribution of molecular forms of estrogen receptors in 443 specimens of both primary and metastatic breast carcinoma determined by sucrose gradient centrifugation As shown the cytosols of 26% of primary breast tumors contained the 8-9S components whereas only 11% of the metastatic lesions exhibited these entities Approximately 20% of either primary or metastatic breast specimens contained both the 8-9S and 4-5S species of estrogen receptors Examination of cytosols revealed that although only 12% of primary breast tumors

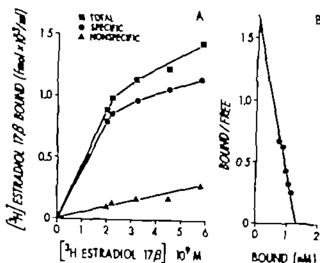


Fig 5 Titration of estrogen-binding sites in a breast carcinoma from a male patient Cytosol containing approximately equal quantities of the 8-9S and 4-5S species (see Fig 4A) was titrated with increasing concentrations of [³H]estradiol-17β in the presence (triangles) and absence (squares) of 250-fold excess of unlabeled diethylstilbestrol (A) Scatchard analysis of specific binding data (B) gave a K_d= 6 X 10^{-10} M number of binding sites was 98 fmoles/mg cytosol protein

contained the 4-5S species, 21% of metastatic breast lesions exhibited these components. In both cases, 40-50% of the breast specimens examined did *not* contain estrogen receptor species.

The estrogen-binding capacities of 326 specimens of primary breast cancer ranged between 7 and 481 fmoles/mg protein with a mean value of 90 ± 94 (Table 3). The levels of estrogen receptors in metastatic breast carcinoma were similar to those observed in primary tumors; the mean value was 76 ± 86 fmoles/mg protein with a range between 8-379 fmoles (Table 3). The values presented in Table 3 for the binding capacities of the various forms of the estrogen receptor were calculated from the same experiments reported in Table 2. The average of the binding capacities for tissues containing both forms of the estrogen receptors was 2 to 3 times greater than the mean value for specimens containing either the 8-9S or 4-5S only. The significance of this result is unknown presently.

Table 2. Distribution of molecular forms of estrogen receptors in human breast cancer[a]

Peceptor species	Primary breast carcinoma	Metastatic breast carcinoma
8-9S	85/326 (26%)	13/117 (11%)
8-9S and 4-5S	62/326 (19%)	21/117 (18%)
4-5S	40/326 (12%)	24/117 (21%)
Undetectable	139/326 (43%)	59/117 (50%)

[a] Determined by sucrose gradient centrifugation.

Table 3. Specific estrogen binding capacity of molecular forms of estrogen receptors in human breast cancer[a]

Receptor species	Primary breast carcinoma	Metastatic breast carcinoma
8-9S	72 ± 83 (7-348)[b]	51 ± 27 (11-182)
8-9S and 4-5S	142 ± 104 (17-481)	129 ± 112 (26-379)
4-5S	43 ± 51 (8-193)	50 ± 60 (8-258)
Total	90 ± 94 (7-481)	76 ± 86 (8-379)

[a] Values presented were calculated from the same experiments used to determine estrogen receptor distribution (Table 2).
[b] Expressed as fmoles/mg cytosol protein, mean ±SEM (range)

ESTROGEN RECEPTOR DISTRIBUTION ACCORDING TO SITE OF TUMOR METASTASES

Consideration of the sites of breast carcinoma metastases is one of the criteria used in the selection of therapy for the breast cancer patient. In Table 4 the number of metastatic specimens examined and their estrogen-binding capacities have been separated according to the site of appearance. The column on the left in Table 4 lists the normal tissues

studied none of which exhibited significant estrogen-binding capacity The majority of breast cancer metastases obtained in our study originated in skin lymph nodes and nonlactating breast Approximately one-half of these contained estrogen receptors with the binding capacities shown Although estrogen receptors were found in several metastases at other sites at no time have we observed a correlation between the presence of estrogen receptors and the location of these lesions in breast cancer patients

Table 4 Sites of breast carcinoma metastases and estrogen receptor distribution

Tissues[a]	Number of metastases by site containing estrogen receptors	Specific binding capacity[b]
Adrenal gland	2/10	100 ± 379
Bone	2/4	15 ± 56
Breast (nonlactating)	10/22	74 ± 86(14-258)
Colon	1/1	36
Kidney	0/1	
Liver	0/2	
Lung	1/3	18
Lymph node	15/31	94 ± 80(8-253)
Muscle	1/3	17
Omentum	2/2	16 ± 179
Ovary	2/2	13 ± 50
Skin	16/30	89 ± 119(9-354)
Stomach	1/1	241
Thyroid	0/1	

[a] In normal tissues which did not contain metastatic deposits estrogen receptors were uniformly undetectable

[b] Expressed as fmoles/mg cytosol protein; mean ± SEM (range)

TUMOR CELL CONCENTRATION AND ESTROGEN BINDING CAPACITY

Since human breast tumors are heterogeneous with regard to cell type we examined the relationship between estrogen-binding capacity and the proportion of tumor epithelium in a breast biopsy (Fig 6) From these data there does *not* appear to be a correlation between the quantity of estrogen receptors in a breast biopsy and the proportion of tumor epithelium It was noted that numerous tumor specimens containing less than 25% tumor epithelium exhibited very high estrogen binding capacities Secondly these data indicated that the specific estrogen-binding capacities of individual tumor specimens containing the same quantity of tumor epithelium were highly variable As shown the quantity of estrogen receptors in these tumors may vary from an undetectable level to one in excess of 100 fmoles/mg cytosol protein Although

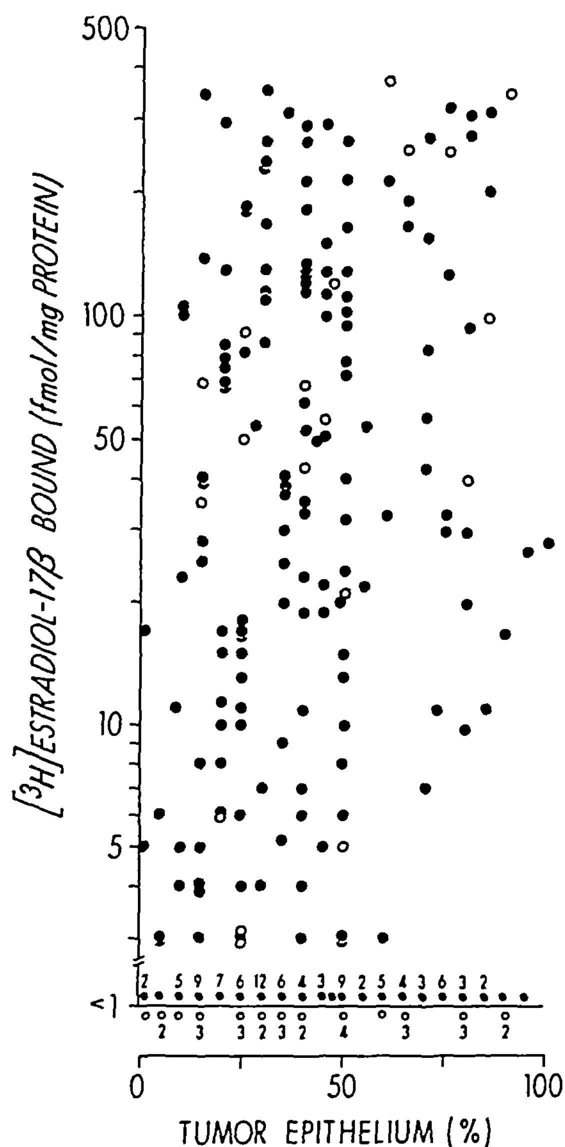


Fig 6. Relationship between specific estrogen-binding capacity and proportion of tumor epithelium in human breast tumors At the time of estrogen receptor analysis, a representative sample of breast tumor was fixed in neutral formalin and processed for histologic examination Relative concentration of tumor epithelium was estimated in specimens of primary (closed circles) and metastatic (open circles) breast carcinoma and compared with estrogen-binding capacity

one may expect that the estrogen-binding capacity of normal mammary gland increases with increasing cellularity, this is not true of breast tumors. Thus, the quantitation differences in estrogen-binding capacity reported earlier (cf. McGUIRE et al., 1975) were not due simply to the quantity of tumor cells in a biopsy. Rather this variation in activity reflects differences in the number of binding sites per breast tumor cell.

Another type of relationship which we have determined is whether a particular type of breast tumor based upon histologic classification of pathology contained a different distribution of estrogen receptors As described earlier biopsies of each breast tumor specimen were prepared for histologic examination at the time of estrogen receptor analyses The pathology of each sample was described using the criteria of FISHER et al (1975) and compared with the type(s) of estrogen receptors observed in the cytosol of the other portion of the tumor used for this analysis As seen in Table 5 approximately 50% of infiltrating ductal carcinomas with pathologies not otherwise specified contained estrogen receptors A greater number of tumors exhibited the 8-9S species in cytosol than the 4-5S estrogen-binding components Various subclassifications of infiltrating ductal carcinoma such as tubular medullary papillary colloid or apocrine carcinoma were also examined As seen a considerable number of specimens classified as tubular carcinomas contained estrogen receptors whereas only two of eleven medullary carcinomas exhibited estrogen-binding capacity Although fewer samples were examined only one of four (described as colloid carcinoma) and none of four apocrine type tumors contained receptors A single specimen of papillary carcinoma contained both the 8-9S and 4-5S species of estrogen receptors Approximately 50% of 13 infiltrating lobular carcinomas also contained receptors; interestingly the cytosols of all seven biopsies examined contained some quantity of the 4-5S forms This was also observed in the samples of intraductal carcinoma Thus with the possible exception of medullary and apocrine tumors there was no differential relationship between the histologic classification of the majority of human breast carcinomas and the distribution of specific molecular forms of estrogen receptors in cytosols At no time have we found significant quantities of estrogen-binding proteins in specimens of chronic mastitis fibrocystic disease, fibroadenoma or gynecomastia of the breast The results of a similar type of study were reported recently (ROSEN et al 1975)

Table 5 Distribution of estrogen receptors and pathology of breast tumors

Histologic classification of carcinomas	Number of specimens containing estrogen receptors[b]		
	8-9S	8-9S and 4-5S	4-5S
Infiltrating ductal (NOS)[a]	72/350	68/350	40/350
Tubular	2/12	5/12	1/12
Medullary	1/11	1/11	0/11
Colloid	1/4	0/4	0/4
Papillary	0/1	1/1	0/1
Apocrine	0/4	0/4	0/4
Infiltrating lobular	0/13	2/13	5/13
Intraductal	0/9	3/9	2/9

[a] Not otherwise specified
[b] For each tumor type the denominator represents the total number of specimens examined (e g 72 of 350 infiltrating ductal carcinomas (NOS) had 8-9S while of the same 350 68 had 8-9S and 4-5S

CLINICAL SIGNIFICANCE OF ESTROGEN RECEPTORS

During the summer of 1974 the National Cancer Institute assembled scientists and clinicians of twelve laboratories from various parts of the world who had both biochemical and clinical data concerning the relationship between a breast cancer patient's response to hormone therapy and the presence of estrogen receptors in a tumor biopsy. The majority of the clinical results also were examined independently by two medical oncologists designated by the National Cancer Institute to ensure that the objective remissions were judged by similar clinical trial criteria. The results of this International Workshop are summarized in Table 6. Details of the individual contributions are contained in a volume entitled Estrogen Receptors in Human Breast Cancer, edited by McGUIRE et al. (1975). The results suggested that, regardless of the type of hormone therapy selected, i.e., endocrine organ ablation or hormone administration, approximately 55% of patients whose tumors contained estrogen receptors responded to these therapies. If the tumor biopsy did *not* contain estrogen receptors, only 8-11% of patients responded to hormone therapy. These results are convincing in spite of a knowledge that these 12 laboratories used several different methods of estimating estrogen receptors and categorized estrogen-binding capacities using different criteria. From the results of the conference it appears that analyses of estrogen receptors in biopsies of breast carcinoma are useful in the selection of hormone therapies for the patient with advanced breast cancer.

The results presented in Table 6 indicated that regardless of the kind of endocrine manipulation, approximately 45% of the patients whose tumors contained estrogen receptors did *not* respond objectively. During the past two years we have concentrated on the question of why these patients whose tumors possess estrogen-binding capacity fail to respond to hormone therapy. It is reasonable to assume that some of these patients whose tumors contain estrogen receptors were so critically ill that they were unresponsive not only to endocrine manipulation, but to other forms of therapy such as cytotoxic agents However, not all of the cases may be explained on this basis.

Although a target cell contains the biologic capacity to associate with steroid hormones, as designated in Figure 1, this property does not obligate the cell to respond to the hormone. As seen from Figure 1, there are numerous additional intracellular events which must be *intact* for the steroid hormone to bring about growth responses in the cell. Recent data from our laboratory (WITTLIFF et al., 1976) suggest that there may be an intracellular marker indicative of a particular defect beyond the initial binding of steroid to the recep[illegible] e. Earlier it was shown that certain human breast tumors only the 8-9S species of estrogen receptors, but oft[illegible] sedimenting at 4-5S was observed. The distribution o[illegible] lecular forms of these estrogen-binding components [illegible] tified in primary and metastatic breast carcinomas [illegible]

CLINICAL SIGNIFICANCE OF MOLECULAR FORMS OF

Because our early studies were retrospe[illegible] clinical responses to hormone therapy o[illegible] tients on whom we have measured estrog[illegible] results were collected on 50 patients [illegible] an objective remission in 29 patients w[illegible]

Table 6 Relationship between estrogen binding capacity of breast carcinomas and objective regressions after hormone therapy

Therapy	Objective remissions according to estrogen binding capacity	
	Estrogen receptor positive	Estrogen receptor negative
Hormone administration	59/105 (56%)	12/109 (11%)
Endocrine organ ablation	59/107 (55%)	8/94 (8%)

[a] Compiled from McGUIRE et al (1975)

Table 7 Relationship between response to endocrine therapy and presence of estrogen receptors in tumor

Therapy[a]	Objective remissions according to estrogen receptor species in tumor[b]		
	8-9S 8-9S and 4-5S	4-5S	Undetectable
Oophorectomy	0/2	0/2	0/4
Adrenalectomy	2/2	0/2	0/4
Estrogen	4/4	0/3	0/10
Androgen		0/1	0/7
Elipten	1/1	0/1	0/4
	7/9	0/9	0/29

[a] Three additional patients exhibited remissions but had unclassified ER positive tumors; two responded to estrogen and one to androgen therapy
[b] Eastern Cooperative Oncology Group criteria were used

who had an estrogen receptor negative tumor regardless of the type of hormone therapy administered This is a far better correlation than reported for the collective results presented at the International Workshop mentioned earlier (McGUIRE et al 1975) We attribute this principally to our use of both the sucrose gradient and dextran-coated charcoal procedures which permit confirmation of steroid binding measurements However 10 of 21 patients whose breast carcinomas contained estrogen-binding capacity responded to a number of endocrine therapies (Table 7) The mean ± SEM of the estrogen-binding capacity of the tumors from patients responding to hormone therapy was 104 ± 43 fmoles/mg cytosol protein With regard to objective remissions in patients who had tumors containing estrogen receptors of the 8-9S type we have observed seven of nine patients who responded to hormone therapy The majority of these tissues had predominantly the 8-9S species with a varying amount of 4-5S receptors As shown by the footnote to Table 7 three additional patients responded to hormone therapy whose tumors contained specific estrogen-binding capacity However because of the type of determination used we were unable to distinguish whether these tumors contained either the 8-9S or the 4-5 estrogen-binding components Interestingly *none* of nine patients responded who were administered hormone therapy and whose tumors contained principally the 4-5S estrogen receptor species

As described earlier in Table 6, approximately 45% of these patients had breast tumors containing estrogen receptors but did *not* respond to these hormonal manipulations by clinical trial criteria. This is not unreasonable since breast cancer is often a complicated disease likely to contain both hormone responsive (dependent) and hormone insensitive cells. Furthermore, since the sequence of intracellular events in the interaction of the steroid hormone with the target cell is manifold, one would predict that defects may also arise at sites other than the loss of the receptor molecule itself (Fig. 1). Whether the 4-5S species of estrogen receptor - an entity uncommonly observed on sucrose gradients of low ionic strength - represents an accumulation of the cytoplasmic steroid-receptor complex due to a defect in a later event, such as activation, is not know presently. Several possibilities concerning their origin have been suggested (WITTLIFF and SAVLOV, 1975).

INFLUENCE OF IONIC STRENGTH ON ESTROGEN RECEPTORS

To determine if the ionic strength of the buffer used to extract estrogen receptors from breast carcinomas influenced their sedimentation properties, tumors were homogenized in Tris buffer in the presence of various concentrations of KCl and separated on sucrose gradients of the same ionic strength. Representative profiles are presented in Figure 7.

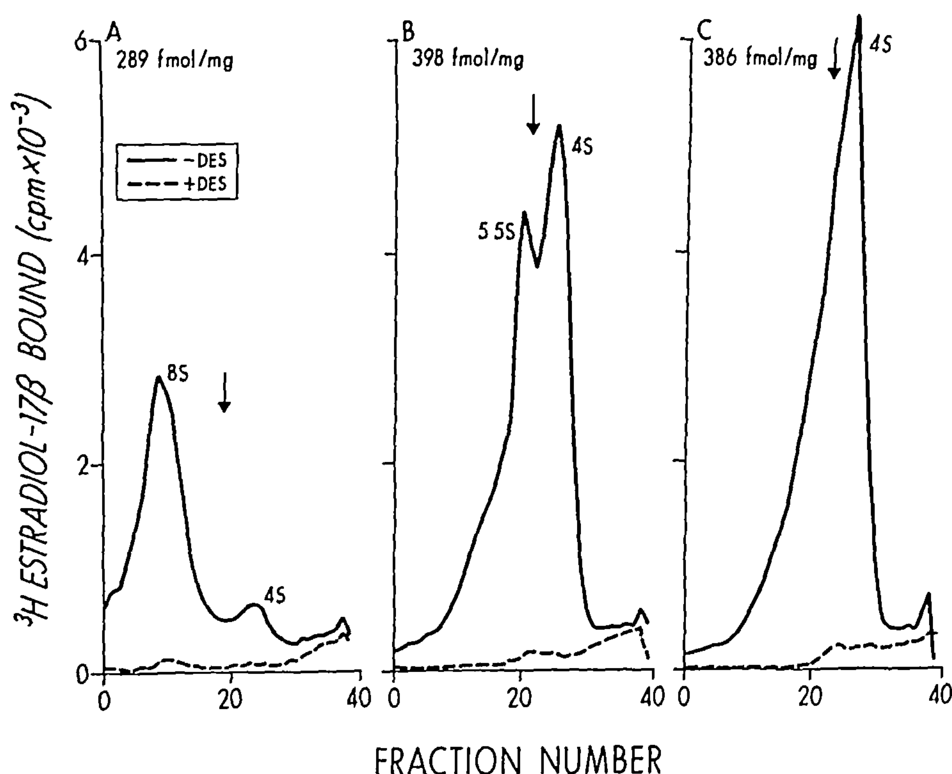


Fig 7 Influence of ionic strength on estrogen receptors in human breast cancer A single specimen of infiltrating ductal carcinoma was minced and divided into portions which were extracted with standard Tris buffer containing either no (A), 0.15M (B), or 0 4M KCl (C) Estrogen receptors were then separated by sucrose gradient centrifugation Linear gradients of either 5-40% (A) or 5-20% (B and C) sucrose were used, sedimentation position of a 4 3S marker is shown by arrows (Taken from WITTLIFF et al , 1976)

As shown in Figure 7A a cytosol containing predominantly the 8-9S species was observed on sucrose gradients containing low ionic strength; this particular preparation contained a specific estrogen-binding capacity of 289 fmoles/mg cytosol protein When a second portion of this same tumor specimen was extracted with 0 15M KC1 and sedimented on gradients containing 0 15M KC1 the profile shown in Figure 7B was observed Note that two species of estrogen receptors were detected one sedimenting at 5 5S and a second sedimenting at approximately 4S In this preparation the specific estrogen-binding capacity increased to 398 fmoles/mg cytosol protein Finally when a third piece of this same tumor specimen was extracted with Tris buffer containing 0 4M KC1 (final concentration) and separated by sucrose gradient centrifugation a single peak with somewhat broad sedimentation properties was observed with a maximum at approximately 4S The estrogen-binding capacity was similar to the profile shown in Figure 7B i e 386 fmoles/mg cytosol protein In addition to the differences observed under conditions of increasing ionic strength we also have detected the presence of a 5 5 S species in addition to the 4S type in a limited number of estrogen receptor profiles obtained under conditions of low ionic strength (data not presented) Similar results indicating the presence of an estrogen-binding component sedimenting at 5-6S on sucrose gradients containing 0 15M KC1 have been observed using cytosol from the lactating mammary gland of the rat (BOYD and WITTLIFF unpublished) When tumors containing only the 4-5S species of estrogen receptors on sucrose gradients of low ionic strength were examined under conditions of 0 15M and 0 4M KC1 no alteration in the sedimentation profiles was observed nor was there an increase in the estrogen-binding capacity

MOLECULAR BASIS OF UNRESPONSIVENESS

As mentioned earlier activation of the cytoplasmic form of the estrogen receptor appears to be a prerequisite for nuclear translocation and chromatin interaction JENSEN et al (1971b) described a cell-free system for transformation of the 4S species to a 5S form of estrogen receptor in rat uterus The properties of this 5S species were similar to those of estrogen receptors isolated from nuclei following temperature-dependent translocation Recent evidence suggests that the molecular basis of the 4S to 5S transformation in uterine cytosol is a dimerization of a 4S component with a second subunit of the receptor (LITTLE et al 1973; NOTIDES and NIELSEN 1974) An alternative explanation has been offered by BRESCIANI et al (1974) suggesting the action of a receptor-transforming factor in uterus Similar results have not been reported for normal or neoplastic breast tissues

The ligand binding affinities and specificities of the 8-9S and 4-5S forms of estrogen receptors were similar in specimens of human breast carcinoma What then is the molecular basis for unresponsiveness in tumors containing estrogen receptors? We *propose* that the estrogen receptor of normal mammary cells and hormonally responsive breast tumors is composed of subunits which combine in the presence of estradiol-17B under physioligic conditions to form a 6S dimer as diagrammed in Figure 8B This entity then translocates into the nucleus and stimulates a cellular response This proposal is supported by the observations that (1) only tumors containing 8-9S estrogen receptors under conditions of low ionic strength yielded the 5 5S species when extracted with buffers of physiologic ionic strength and (2) only patients with breast tumors exhibiting 8-9S estrogen receptors have responded to hormonal therapy Thus certain unresponsive breast tumor cells apparently do not contain a full complement of estrogen receptors necessary

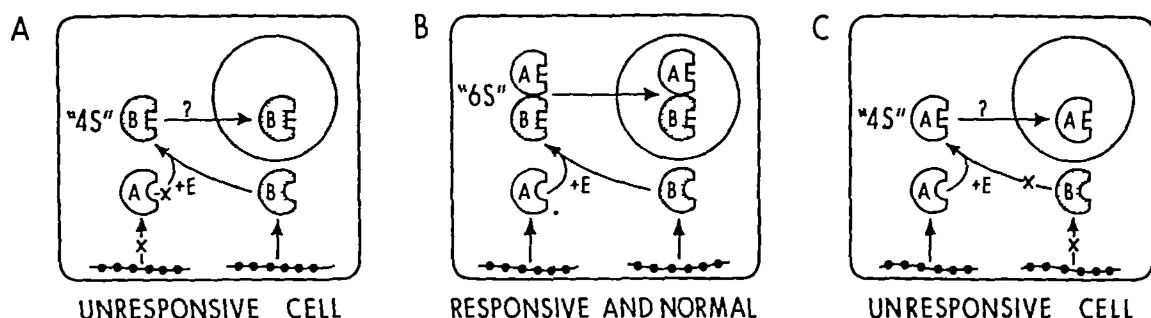


Fig 8 Proposed explanation of unresponsiveness in estrogen receptor positive breast cancer patients Subunits of estrogen receptor in normal and responsive breast cells (B) are depicted as separate entities which sediment at "4S" In the presence of estradiol-17ß, the subunits combine to form a "6S" dimer which then translocates into the nucleus and stimulates cellular response. The "6S" components usually aggregate in vitro to the 8-9S forms of estrogen receptors commonly observed on sucrose gradients of low ionic strength In unresponsive cells (shown in A and C), two types of defects shown by the X are postulated, either of which results in detection of "4S" components only (Taken from WITTLIFF et al , 1976)

for activation. Presumably a single estrogen-binding subunit of the 4S type is synthesized by these cells (Figs. 8A,C). The other subunit which presumably also sediments at 4S is either not synthesized or does not combine properly with the second 4S component. Thus, only a 4S species of estrogen receptor is detected in cytosol when these breast tumors are examined by sucrose gradient centrifugation.

In summary, our results suggest that the molecular properties and behavior of estrogen receptors in human breast tumors are related to the clinical responsiveness of patients given hormonal therapy Since the 8-9S and 4-5S species are *not* distinguished by procedures such as the dextran-coated charcoal method, Sephadex G-25 chromatography, and the DEAE filter technique (see WITTLIFF, 1975), we recommend that separation procedures such as sucrose gradient centrifugation be used until possible differences in the molecular behavior of estrogen receptor species in human breast carcinomas have been elucidated

EPILOGUE

At the conclusion of Dr. Elwood V. Jensen's presentation at the Laurentian Hormone Conference in 1961 (JENSEN and JACOBSON, 1962), during which he presented some of the initial evidence on the binding of tritium-labeled estradiol-17ß in hormonally responsive organs, Dr. Gilbert S. Gordan inquired:

Elwood's steroids are ever so hot,
But there's one thing we still haven't got.
Through the liver they zoom
While they stick to the womb.
In the breast, can you tell us what's what?

to which Dr. Jensen replied:

To put Dr. Gordan at rest.
The difficult thing about breast
Is the lack of amounts
Of significant counts
So our answers are not of the best.

Ten years later when the author and co-workers (WITTLIFF et al 1972a b) first separated the specific estrogen-binding proteins from lactating mammary gland using sucrose gradient centrifugation David G Gardner and the author sent the following response to Dr Jensen:

The problems you encounter with breast
Depend on the stage which you test
With hormone stimulation
and cell proliferation
It ll bind just as well as the rest

Investigations by a multitude of scientists have provided insight into the specific temporal sequence by which a steroid hormone stimulates the orderly differentiation and growth of the mammary gland (cf WITTLIFF, 1975) The original suggestion of JENSEN et al (1967) that the ability of a breast carcinoma to bind estrogen may be predictive of a patient s response to hormone therapy has now been supported wide (cf McGUIRE et al 1975) Therefore it is evident that the analysis of estrogen receptors in a breast tumor biopsy must be included with response to previous hormone therapy age and menopausal status site of the dominant metastatic lesion and disease-free interval as criter for the selection of endocrine therapy for the patient with advanced breast cancer

ACKNOWLEDGMENTS

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Chapter 8
Biochemical Markers in Cancer of the Breast

D C TORMEY and T P WAALKES

INTRODUCTION AND METHODS

This report will highlight the current approaches being used in the development of biochemical marker tests for breast carcinoma. Available data with selected tests will be used to aid in illustrating the "state of the art."

The modern development of tumor-associated biochemical markers received considerable impetus with the demonstration of carcino-embryonic antigen (CEA) (GOLD and FREEDMAN, 1965). Since that time CEA has been demonstrated to be abnormally elevated in the blood of many patients with different types of cancer, as well as in some selected nonneoplastic conditions (LO GERFO, et al., 1971); REYNOSO et al., 1972 and CONCANNON et al , 1973). As a prototype for tumor-associated biochemical markers, CEA constitutes a very different type of marker than do the steroid receptor proteins The steroid hormone receptors appear to provide a therapeutically selective marker, i.e., their presence or absence in the tumor provides the clinician with a tool to aid in the decision of whether or not to consider hormone therapy for the individual patient. In contradistinction, the major characteristics of an ideal biochemical marker for breast cancer would include: (1) the sensitivity to detect all patients with the disease in the absence of any evidence by standard clinical or laboratory tests; (2) high specificity in order to detect only those patients with the disease, (3) the capacity to quantitatively follow the actual tumor burden of the host, i.e., the level should increase with progressive disease, and should decrease as the disease enters remission; (4) the capability to quantitatively detect disease below the level of current detection methods, i.e., when there are $<10^{10}$ tumor cells in the patient, and preferably at levels well below 10^6cells; and (5) the mechanics of the test should enable it to be economically integrated into most hospital clinical laboratories.

Since no current test has the desired specificity, nor detects the presence of disease in all patients, one approach to the problem is to ascertain if the detection rate could be enhanced through the use of a matrix of tests similar to the fashion in which one evaluates hepatic damage. The conceptual basis for this approach is depicted in Figure 1. The initial screen with a proposed new marker is performed on samples from patients with metastatic disease. The test is assessed in terms of its specificity, its detection capability, whether or not it follows the clinical tumor burden, and whether or not it either adds to the matrix of those tests already available or should replace one or more of the tests in the current matrix If the test still looks promising it is then screened alone and within the matrix using samples from post-operative patients with histologic evidence of involvement in

Fig 1 Proposed sequence of clinical testing in evaluating a potential biochemical marker in breast carcinoma

1 Ascertain detection capability and tumor burden relationship in metastatic disease

2. Ascertain detection capability in postoperative N+ disease and in preoperative disease.

3 Test predictive capability in postoperative N disease and in screening setting

the resected axillary nodes i e N+ patients In this setting the test s role is assessed to ascertain if it adds significantly to the detection rate under conditions of minimal disease where the majority of patients will relapse in a relatively short time If the individual test and the matrix still appear promising then postoperative patients without axillary node involvement i e N- patients are sequentially screened to ascertain if the components of the test matrix and the matrix proper will predict which patients will show recurrences

The tests utilized in our initial screen at NCI are shown in Table 1 The normal values and units of measurement for the assays are also shown The polyamines putrescine (PUT) spermidine (SPD) and spermine (SPM) were chosen because they had previously been found elevated in the urine of patients with a variety of neoplasms other than breast cancer and are frequently found to be increased in settings involving redifferentiation or rapid growth (RUSSEL 1971; DENTON et al 1973; WAALKES et al 1975a) The putrescine to spermidine to spermine metabolic pathway was also assessed by using product-precursor ratios The polyamines, which also include cadaverine (CAD) were detected in 24

Table 1 Biochemical marker tests utilized in initial NCI screen

Test	Mean	Range[a]	Units
PUT	0 42	0 11 - 0 73	μM/kg/24 hrs
SPD	0 110	0 042 - 0 178	μM/kg/24 hrs
SPM	0 010	0 000 - 0 020	μM/kg/24 hrs
SPD/PUT	0 044	0 038 - 0 550	-
SPM/PUT	0 024	0 000 - 0 052	-
SPM/SPD	0 090	0 000 - 0 192	-
CAD	0 055	0 000 - 0 218	μM/kg/24 hrs
ψ	0 78	0 37 - 1 19	mg/kg/24 hrs
m_2^2G	0 06	0 01 - 0 11	mg/kg/24 hrs
m I	0 05	0 00 - 0 10	mg/kg/24 hrs
hCG	-	0 - 5	mIU/ml
CEA	1 7[b]	0 7 - 2 7[b]	ng/ml

Normal mean and units of measurements are indicated [a] = ± 2 SD about the mean [b] = nonsmokers only; normal value for smokers is ≤ 5 ng/ml PUT = putrescine SPD = spermidine SPM = spermine CAD = cadaverine ψ = pseudouridine m_2^2G = N^2 N^2-dimethylguanosine m I = 1-methylinosine hCG = human chorionic gonadotrophin CEA = carcinoembryonic antigen

hour urines using an amino acid analyzer (GEHRKE et al., 1974) The minor nucleosides were chosen because they also have been found elevated in a variety of malignant diseases (ADAMS et al., 1960; PINKARD et al., 1972; WAALKES et al., 1975b), although they had not as yet been investigated in patients with breast cancer. They are predominantly minor transfer RNA nucleosides whose synthesis is completed after the transfer RNA molecule is produced. Upon degradation of transfer RNA pseudouridine (ψ) is neither metabolized nor reincorporated; its level has been considered to reflect transfer RNA turnover (WEISSMAN et al., 1962). The levels of the methylated nucleosides N^2, N^2-dimethylguanosine (m_2^2G), and 1-methylinosine (m'I) are felt to reflex the activity of transfer RNA methylases (RIDDICK and GALLO, 1970; WAALKES et al., 1971). These enzymes have been shown to be present in abnormally high levels in many tumors (TSUTSUI et al, 1966). The nucleosides were measured by gas chromatographic techniques in 24 hour urines collected under purine restricted dietary conditions (CHANG, et al, 1974) Plasma CEA and serum human chorionic gonadotrophin (hCG) were chosen because both had previously been shown to be elevated in some patients with breast cancer (LO GERFO et al., 1971; REYNOSO et al, 1972; CONCANNON et al, 1973; BRAUNSTEIN et al., 1973). They were measured by radioimmunoassay techniques (HANSEN et al., 1974; VAITUKAITIS et al, 1972). Portions of the data to be presented concerning these nine tests have been previously published (WAALKES et al., 1975a; WAALKES et al., 1975b, TORMEY et al., 1975).

RESULTS AND DISCUSSION

Incidence of Elevated Tests

The nine tests, and the polyamine ratio calculations, were performed on 69 patients with metastatic disease prior to the initiation of a therapeutic regimen The proportion of patients with elevated results varied from 1.4% for Putrescine to 65.2% for CEA (Table 2) Single marker elevations occurred only with the tests for hCG, N^2, N^2-dimethylguanosine, and CEA Of the 69 patients, 68, or 98.6%, had elevations of one or more of the nine tests

Further analysis of the data in Table 2 revealed that 92 8% (64/69) of the patients had elevated levels of CEA, hCG, and/or N^2, N^2-dimethylguanosine (Fig. 2) Considering only these three tests the only elevated marker in 10 1% of the patients was hCG; N^2, N^2-dimethylguanosine was the only abnormality in 15.9% and CEA in 20.3%. All three tests were elevated in 11 6% of the patients. No additional patients were detected by considering cadaverine, pseudouridine, or 1-methylinosine levels. The elevated tests in the remaining four patients involved the polyamine ratios and spermidine and spermine. Since the polyamines only detected an additional 5.8% of the patients these tests were not considered to be particularly useful in the detection matrix at the present time.

The three test matrix of CEA, hCG, and N^2N^2-dimethylguanosine was evaluated in 26 postoperative patients who were under observation between 3 weeks and 6 months after primary local therapy. None of these patients had clinically detectable disease Elevations of one or more tests were present in 12, or 46% of the cases. One additional patient had a pseudouridine elevation, and two additional patients had polyamine elevations

Among 13 preoperative patients, 7, or 54%, had abnormalities involving the CEA, hCG, and N^2, N^2-dimethylguanosine matrix. Two additional

Table 2 Incidence of elevated tests in 69 patients with metastatic breast carcinoma

Test	Total number (%) Elevated	Single number (%) elevated Without ratios[a]	With ratios
PUT	1 (1 4)	0	0
SPD	18 (26 1)	1 (1 4)	0
SPM	17 (24 6)	2 (2 9)	0
CAD	3 (4 3)	0	0
ψ	15 (21 7)	0	0
m_2^2G	26 (37 7)	3 (4 3)	3 (4 3)
m I	14 (20 3)	0	0
hCG	33 (47 8)	3 (4 3)	3 (4 3)
CEA	45 (65 2)	6 (8 7)	4 (5 8)
SPD/PUT	29 (42 0)	1 (1 4)	0
SPM/PUT	24 (34 8)	1 (1 4)	0
SPM/SPD	14 (20 3)	0	0
All tests normal		2 (2 9)	1 (1 4)

[a] In ratio lines this is without considering other ratios

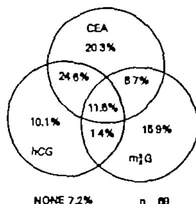


Fig 2 Interaction between CEA hCG and m_2^2G elevations in metastatic breast carcinoma

patients had polyamine elevations Thus a single analysis with the three test matrix detected abnormalities in 54% of the preoperative patients 46% of the 1-6-month postoperative patients and 93% of the patients with metastatic disease

Relationships to Tumor Burden

Correlations between the tumor burden and the quantitative levels of a marker can be sought across either patient populations or individual patients The incidence of elevated levels can also be correlated across populations of patients with differing tumor burdens A major assumption in these analyses is the clinical tumor burden definition One approach to estimating this is to define the tumor burden as being lowest in the postoperative state and progressively increasing through the preoperative state to the metastatic state In addition the

average tumor burden could be considered to be increasing in the metastatic state with increasing numbers of organ sites of involvement; thus, a patient with three organ sites of involvement would generally be considered to have a higher tumor burden than a patient with one organ site of metastatic involvement By pooling the data for all patients within each tumor burden classification, it is then possible to estimate whether or not the marker test results correlate with the tumor burden in populations of patients. Correlations observed across populations of patients in accordance with this analysis would generally be expected to be seen in individual patients. If they are not one must be concerned about the validity of the tumor burden definition. Alternative explanations for a lack of correlation could include unexpected factors in either the assay procedure or in the biology of the marker with reference to the host.

Using the above population approach two general patterns have emerged The data for CEA exemplifies one of the patterns (Table 3). As the tumor burden increases the incidence of elevated values increases from 7.7% to 80.4%, and the mean quantitative level increases from 1.5 ng/ml to 68.81 ng/ml. At high tumor burdens of three or more organ sites of involvement the proportion of patients with elevated levels and the mean quantitative value decrease. The decrease at high tumor burdens has been associated with a poor prognosis in lung and colon carcinomas (WAALKES, 1975c; RAVRY et al., 1974). The reasons for this latter association are presently not clear. Overall this analysis suggests that CEA levels reflect the relative tumor burden of the patient populations. This general type of pattern was also observed for hCG, pseudouridine, and cadaverine.

The pattern of the remaining five tests was a continuous trend toward an increasing incidence and quantitative level from the lowest to highest clinical tumor burdens, again suggesting that the level of the tests may be related to the tumor burdens in these populations of patients.

A modification of this population approach is to examine the proportion of patients with metastatic disease who have elevated levels prior to therapy, during a response, and after progression or relapse (Table 4). This analysis shows that the incidence of elevated values is lower for all the tests, except putrescine, during a response than it is prior to therapy or during progressive disease or relapse. These observations suggest that the marker values tend to follow the clinical tumor burden in populations of patients under therapy for metastatic disease.

Table 3 Relationship of incidence of elevated CEA levels and mean quantitative levels of CEA to clinical state of patients with breast carcinoma

Patient subset	Number of patients	% elevated	Mean value (ng/ml)
Post-op	39	7 7	1 5
Pre-op	14	14 2	2 8
Met - 1 site	36	72.2	57 05
- 2 sites	46	80 4	61 04
- 3 sites	17	58 8	68 81
->3 sites	15	46 7	38 87

Table 4 Incidence of elevated test results and clinical state of patients with metastatic breast carcinoma

Test	Number elevated/total (%)		
	Pretherapy	Response	PD or REL
PUT	2/45 (4 4)	2/28 (7 1)	2/17 (11 8)
SPD	11/45 (24 4)	6/28 (21 4)	10/17 (58 8)
SPM	10/45 (22 2)	0/28 (0 0)	5/17 (29 4)
CAD	1/39 (2 6)	0/22 (0 0)	1/17 (5 9)
SPD/PUT	14/45 (31 1)	5/28 (17 8)	9/17 (52 9)
SPM/PUT	15/45 (33 3)	3/28 (10 7)	6/17 (35 3)
SPM/SPD	8/45 (17 8)	1/28 (3 6)	2/17 (11 8)
ψ	8/39 (20 5)	0/28 (0 0)	4/18 (22 2)
m_2^2G	13/35 (37 1)	4/25 (16 0)	5/17 (29 4)
m I	4/36 (11 1)	1/26 (3 8)	5/17 (29 4)
hCG	14/41 (34 1)	4/25 (16 0)	9/16 (56 2)
CEA	22/38 (57 9)	6/23 (26 1)	10/15 (66 7)

PD = progressive disease REL = relapse following period of response to therapy Response refers to ≥ 50% reduction in disease in ≥ 50% of the involved organ sites with no site progressing and no new lesions appearing Samples from patients receiving chemotherapy were obtained 10-14 days after preceding dose in the study and in those studies noted in Figures 3-8

Individual patients with metastatic disease have had sequential marker assays performed across therapeutic result categories in an attempt to ascertain whether or not the quantitative levels of the markers parallel the course of the disease In Figure 3 the left-hand panel shows that elevated levels of CEA tended to decrease with the attainment of a response in 15 patients Conversely elevated levels rose with the development of progressive disease The right-hand panel shows that normal levels tended to follow the same pattern

Individual patients with data extending over several clinical categories are shown in Figure 4 Again the level of CEA appeared to parallel the clinical course of the disease across the categories from pretreatment to no change improvement response and progressive disease or relapse Similar results have been reported by STEWARD et al 1974 and *CHU and NEMOTO* 1973 for CEA

Figure 5 shows similar clinical correlations with hCG, i e an elevated level tends to drop with the attainment of a response, and the level tends to increase with the development of a relapse or progressive disease Results across three or more clinical classifications in individual patients were also similar to that observed with CEA

In contradistinction to the results with CEA and hCG the correlation across clinical result categories in individual patients with the three nucleosides is less clear Figure 6 shows the results with pseudouridine The results with this test which appear to be similar to those with the other nucleosides suggest a poorer correlation of the posttreatment levels with the clinical state If these results are maintained with larger series one interpretation would be that the therapeutic disturbance of the host and tumor milieux renders the test of

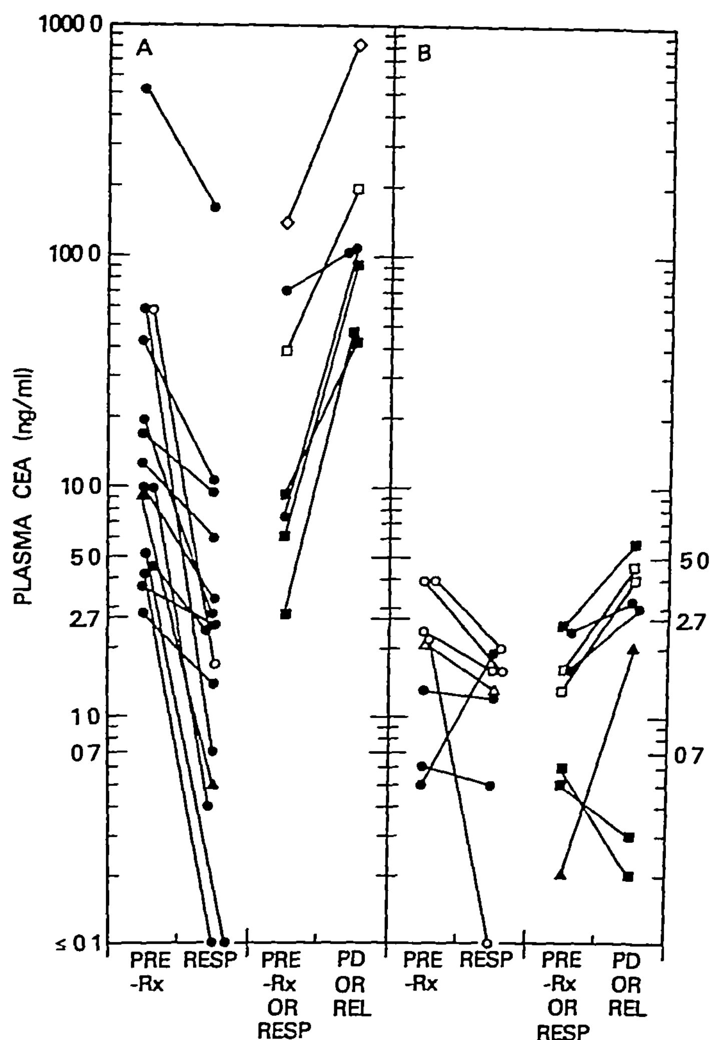


Fig 3. Changes in CEA levels during two sequential clinical states in individual patients with metastatic breast carcinoma A Patients starting from elevated CEA values B Patients starting from normal CEA levels PRE Rx = pretreatment value, RESP = see footnote to Table 4, PD = progressive disease, REL = relapse, closed symbols = nonsmokers, open symbols = smokers, ◇ = smoking history unknown. Initial values were obtained prior to therapry (circles), during a response (squares) or during a period of no change after initiation of therapy (triangles)

less value once therapy is initiated. However, as already alluded to, the test does appear to follow the clinical tumor burden in the therapeutically unperturbed state.

Figure 7 shows sequential clinical results with spermidine, which are representative for the polyamines with the possible exception of cadaverine for which data is currently not available. In general, the levels tended to decrease during a response and increase prior to or in association with progressive disease. Of interest, however, is that in some patients the values increased prior to progressive disease. This appeared to occur up to 100 days prior to the progression. Similar results are shown for spermine in Figure 8. These observations suggest that the Putrescine to Spermidine to Spermine polyamine pathway might be useful for predicting relapse up to several months prior to the clinically observed event in selected patients.

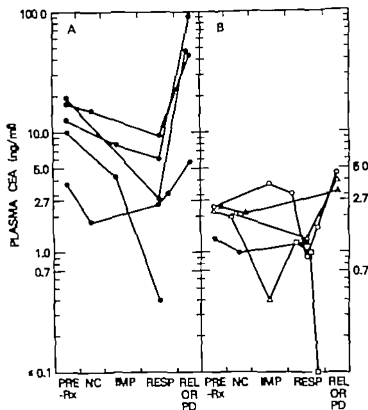


Fig 4 Changes in CEA levels obtained across three or more clinical response categories in patients with metastatic breast carcinoma All patients were treated with combination chemotherapy A Patients with elevated pretreatment CEA levels B Patients with normal pretreatment CEA levels PRE Rx = pretreatment NC = no change IMP = subjective improvement of disease-related bone symptoms RESP = see footnote to Table 4 REL = relapse PD = progressive disease closed symbols = nonsmokers open symbols = smokers Patients were treated with chemotherapy (circles) hormone therapy (triangles) or hormone therapy plus chemotherapy (squares)

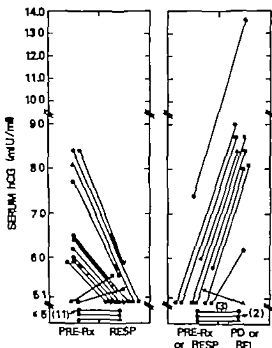


Fig 5 Changes in hCG levels during two sequential clinical states in individual patients with metastatic breast carcinoma Numbers in brackets identify number of patients with indicated sequence of results PRE Rx = pretreatment RESP = see footnote to Table 4 PD = progressive disease REL = relapse closed symbols = first test was pretreatment open symbols = first test was during a RESP Patients were treated with chemotherapy (circles) oophorectomy (squares) oophorectomy plus chemotherapy (■) or diethylstilbesterol (triangles)

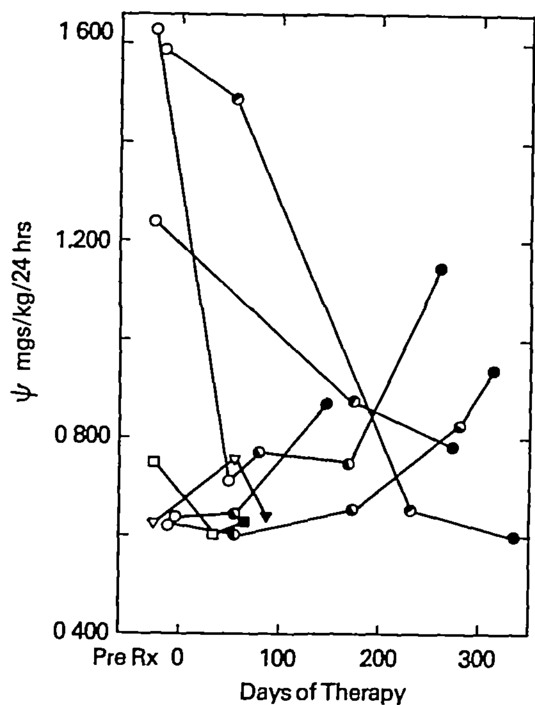


Fig 6 Changes in ψ levels obtained across sequential clinical response categories in patients with metastatic breast carcinoma Open symbols= pretreatment and no change levels, half-closed symbols = levels during response defined in footnote to Table 4, closed symbols = progressive disease or relapse Patients were treated with chemotherapy (circles), exogenous hormones (triangles) or oophorectomy (squares)

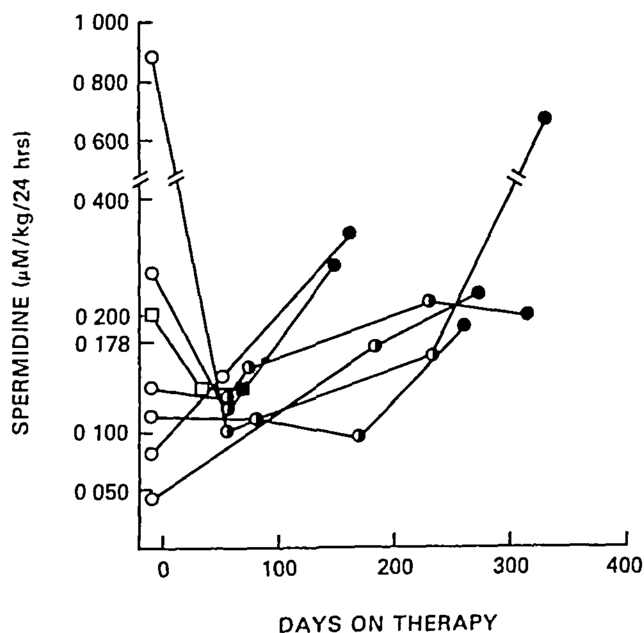


Fig 7 Changes in spermidine levels obtained across sequential clinical response categories in patients with metastatic breast carcinoma Open symbols = pretreatment and no change levels, half-closed symbols= progressive disease or relapse Patients were treated with chemotherapy (circles) or oophorectomy (squares)

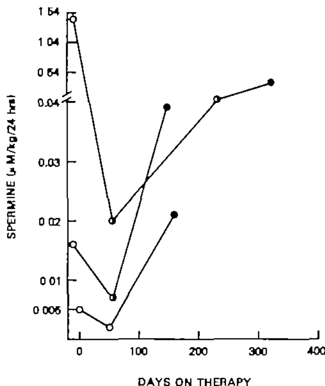


Fig 8 Changes in spermine levels obtained across sequential clinical response categories in patients with metastatic breast carcinoma Each patient was treated with chemotherapy O = pretreatment or no change levels ◐ = levels during response defined in footnote to Table 4 ● = progressive disease or relapse

Prognostic Utility of Biochemical Markers

In patients with metastatic disease it was found that pretherapy polyamine and nucleoside levels were not independently correlated with response rates or the time to therapeutic failure following the institution of chemotherapy However there did appear to be a prognostic relationship between these factors and the pretherapy CEA and/or hCG levels

Figure 9 shows the therapeutic regimens employed in this study Patients were randomly allocated some receiving the cyclophosphamide methotrexate 5-fluorouracil or CMF regimen and others the similar adriamycin-containing or CAF regimen A response to therapy was defined as a 50% or more reduction in disease in at least 50% of the involved organ sites The response rates to these two regimens are shown in Table 5 The adriamycin-containing CAF regimen appeared to be superior in this trial with a higher response rate and a median time to therapeutic failure that was >3 months more than that observed with CMF Prognostic determinants of parameters such as menopausal status performance rating and dominant disease were similar in the two groups

NCI PROTOCOL B122

Cyclophosphamide 100 mg/M^2 p o dl 14
Methotrexate 40 mg/M^2 i v dl and 8
5 Fluorouracil 600 mg/M^2 i v dl and 8

vs

Cyclophosphamide 100 mg/M^2 p o dl-14
Adriamycin 30 mg/M^2 i v dl and 8
5 Fluorouracil 500 mg/M^2 i v dl and 8

Each regimen is repeated every 28 days

Fig 9 Chemotherapy regimens utilized in NCI protocol B122 in patients with metastatic breast carcinoma not previously treated with chemotherapy

Table 5. Response data in patients randomized into NCI protocol B122

Regimen	Eligible	Too early	Response[a]					% CR PR + IMP
			CR	PR	IMP	NC	PD	
CAF	39	2	7	24	1	4	1	86.5
CMF	40	0	2	23	0	6	9	62 5

See Figure 9 for definition of the regimens. CR = complete remission, PR = partial remission, IMP = subjective improvement of osseous disease, NC = no change for > 8 weeks, PD = progressive disease in ≤ 8 weeks.

[a] p = 0.016

Table 6 shows the response rates as a function of the pretherapy CEA levels. A level ≤ 5 ng/ml was associated with responses in 12/12 patients whereas a level of > 5 ng/ml was associated with a 60.9% response rate. Elevated pretherapy hCG levels were also associated with a relatively low response rate (Table 7). Responses were observed in 71.4% of the patients with elevated hCG levels as compared to 94 7% with normal levels. The interaction of these two tests upon the response rate is depicted in Table 8. In general, a low CEA level appears to dominate the effect of the hCG level; however, a normal hCG level appears to improve the response rate associated with an elevated CEA level, raising it from 68.2% to 90.0%. When both tests were > 5 the response rate was only 50.0%.

The impact of the pretherapy CEA level upon the duration of time from the initiation of therapy to therapeutic failure is shown in Figure 10. When the CEA level was >5 ng/ml the duration was shorter than with values ≤5ng/ml ($\underline{P}$ = .038). Elevated hCG levels were also associated

Table 6 Response data with CMF and CAF chemotherapy in metastatic breast carcinoma related to the pretreatment CEA level (in ng/ml)

CEA level	Eval	Response			% Resp	p
		Resp	NC	PD		
≤ 5	12	12	0	0	100 0	
> 5	23	14	4	5	60 9	0 012

Eval = number of patients evaluable, RESP = response, NC and PD = see footnote to Table 5

Table 7. Response data with CMF and CAF chemotherapy in metastatic breast carcinoma related to pretreatment hCG level

hCG level	Eval	Response			% Resp	p
		Resp	NC	PD		
≤ 5	19	18	0	1	94 7	
> 5	21	15	2	4	71 4	0 056

hCG level is expressed in mIU/ml Eval = number of evaluable patients, RESP = response NC and PD = see footnote to Table 5

Table 8 Interaction between pretreatment CEA and hCG levels and response to CMF and CAF chemotherapy in metastatic breast carcinoma

hCG level	CEA level ≤ 5	> 5	UNK
≤ 5	100 0	90 0	94 4
> 5	100 0	50 0	75 0
UNK	100 0	68 2	80 6
			n = 36

The CEA and hCG levels are expressed in ng/ml and mIU/ml respectively Response rates are indicated as percentages UNK = if the test result was unknown

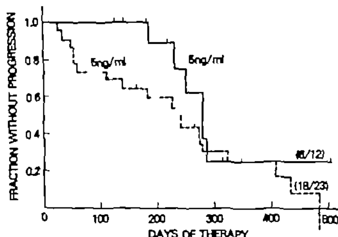


Fig 10 Time from initiation of CMF and CAF chemotherapy to therapeutic failure related to pretreatment CEA level Fractions indicate the number of therapeutic failures/total number of patients in life-table plots

with a shorter duration however the P value was 0 12 These observations while of biological interest are probably not very useful clinically However when both the CEA and hCG levels were >5 the median duration was 111 days shorter than if either or both tests were normal (Fig 11) The P value for these data is 016 (Generalized Wilcoxan test one-tailed) Throughout these response rate and duration analyses the compared groups had similar distributions of other more commonly

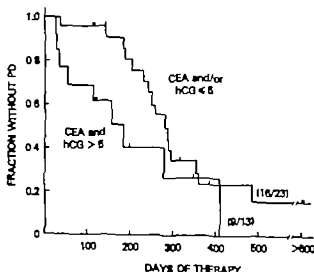


Fig 11 Time from initiation of CMF and CAF chemotherapy to therapeutic failure related to pretreatment CEA and hCG levels Fractions indicate number of therapeutic failures/total number of patients in life-table plots

assessed clinical prognostic variables, including the regimes utilized. It appears, therefore, that the CEA and hCG pretherapy levels may be important prognostic factors for patients treated with these chemotherapy regimens for metastatic disease.

In the preoperative and postoperative groups there were an insufficient number of patients available to reliably estimate the impact of surgery on changes in the marker levels. Figure 12 is reasonably representative and shows little change in the CEA levels when the preoperative value was normal. However, the elevated preoperative levels in two patients did decrease to normal postoperatively.

At the present time there are insufficient data to assess whether or not the preoperative or postoperative levels of any of the markers, or of the matrix, reliably predicts which individual patients will recur early. Among the postoperative patient there were 7 N+ and 1 N- patients who recurred. Five of these patients had preceding elevations of CEA, hCG and/or N^2, N^2-dimethylguanosine. A sixth patient had a preceding elevation of the spermidine/putrescine ratio.

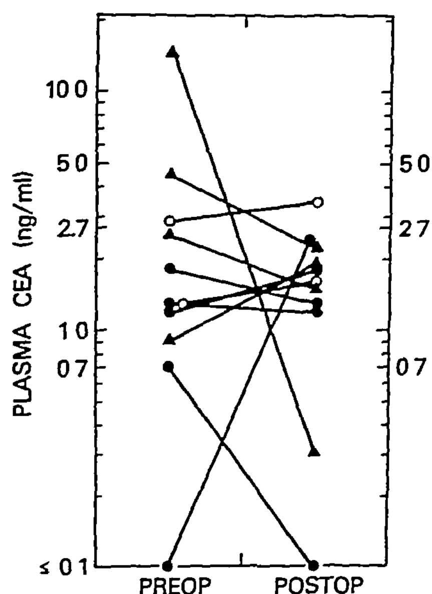


Fig 12 Change in CEA levels from the preoperative to postoperative states Open symbols = smokers, closed symbols = nonsmokers, circles = N- patients, triangles = N+ patients Samples were obtained within 2 weeks prior to operation and 3 weeks postoperative

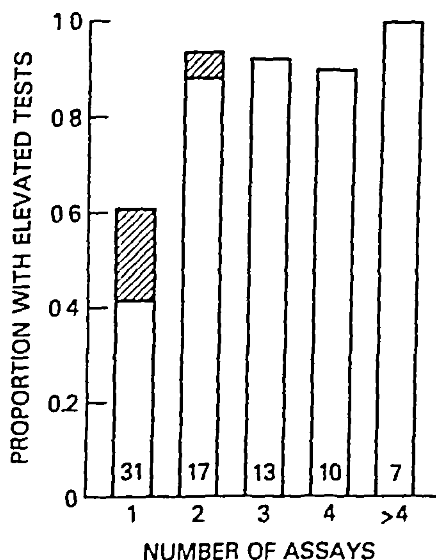
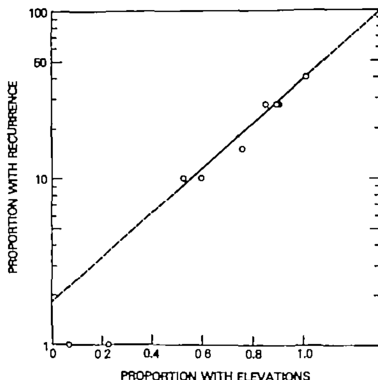
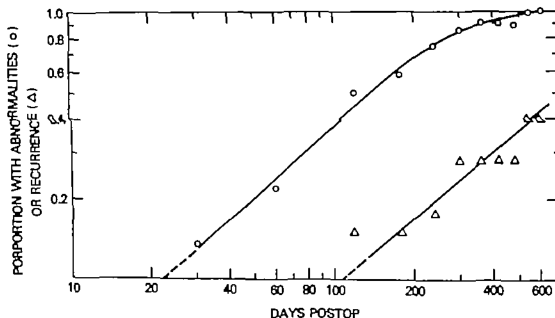


Fig 13 Relationship between number of assays performed postoperatively in N+ patients and the proportion of patients with one or more marker elevations. Each patient's first assay was performed within 12 months from mastectomy No patient had clinical evidence of disease Open bars = proportion of patients with abnormalities of hCG, CEA and/or N^2, N^2-dimethylguanosine, shaded bars = proportion added by also considering remaining six tests and the polyamine ratios. Number at bottom of each bar indicates number of patients with that number of assays



14 Relationship between proportion of postoperative N+ patients h hCG CEA and/or N2 N2-dimethylguanosine elevations and proportion patients with disease recurrence Elevated test results in recurring ents were used only if they preceded recurrence Proportion of rring patients is plotted from a life-table analysis



15 Relationship between time from mastectomy in postoperative patients and proportion of patients with (1) hCG CEA and/or N2 -dimethylguanosine elevations and (2) recurrence Proportion of pa- ents recurring is a life-table plot

Thirty-one postoperative N+ patients had their first assay matrix performed within 12 months from the primary local therapy. The proportion of patients with CEA, hCG, and/or N^2, N^2-dimethylguanosine elevations, or with any elevation, increased from 42% and 61%, respectively, with one analysis, to 88% and 94% with two analyses, and to 100% with four analyses (Fig. 13). The proportion of N+ patients with CEA, hCG and/or N^2, N^2-dimethylguanosine elevations increased in relation to the log of the proportion of patients with a recurrence of disease (Fig. 14), suggesting a functional relationship between the development of an abnormality and a disease recurrence. Figure 15 shows that all of the N+ patients had elevated test results by 600 days postoperatively at which time 40% had recurred. There also appears to be a logarithmic relationship between the time frames at which the patients developed abnormal tests and the proportion of patients with recurrences.

Since the vast majority of the N+ subset of patients will recur, the development of elevated marker values, if they relate to the presence of disease, is not surprising The critical study will be a sequential sampling trial in N- patients. Such a subset will only have a 24% chance of recurring over 10 years; thus, if elevated test results are truly correlated with imminent recurrence the relationship should be detectable in this subgroup.

Of interest is the recent publication by WANG et al., 1975, in the European Journal of Cancer. Their data shows that patients with elevated postoperative CEA levels had a higher recurrence rate than patients with normal levels. The authors correctly pointed out, however, that the analysis did not consider the axillary node status of the patients. Thus, it is possible that the higher recurrence rate in the group with elevated CEA levels was a reflection of an N+ status. Nevertheless, their results are in agreement with ours in that they point to a possible relationship between elevated postoperative test results and early recurrence.

SUMMARY

Currently, one could summarize this area by saying that we appear to be in a situation where three relatively nonspecific tests detect the majority of patients with metastatic disease, as well as those postoperative patients who are at high risk of relapse. The critical test of their utility for segregating those at risk for relapse from those who are not at high risk will need to be done in a highly select subgroup, e.g., N- patients. Two of these tests, CEA and hCG, also appear to be useful indicators for predicting the probability of responding to combination chemotherapy in metastatic disease. The development and further testing of potentially more specific markers to replace or add to the current matrix is now in progress, Casein, which is a product of the milk synthesis pathway of breast tissue, represents a potentially more specific test than any of those studied to date. HENDRICK and FRANCHIMONT, 1974, have found elevated levels in 21 of 26, or 81%, of patients with metastatic disease, and 8 of 11, or 73%, of patients preoperatively The test may also reflect the tumor burden since the proportion of patients with elevated levels dropped to 41-50% postoperatively. Further results with this marker are awaited with interest. Other tests such as ferritin, hydroxyproline, or the development of tumor antigen associated immunospecific assays could increase both the specificity and sensitivity of the tests utilized in this field of investigation. Injecting the use of both single marker tests and matrix approaches into routine clinical use in the postoperative setting now

appears to be ready for more critical testing Their use in diagnostic or screening settings which is the ultimate goal also needs to be evaluated

Finally from the practising clinician s viewpoint the data in this discussion should be considered preliminary It constitutes a status report Although there is evidence that CEA and hCG are prognostic in metastatic disease and that subclinical disease is detectable larger and more tightly controlled studies will be necessary before their routine clinical use can be recommended in breast cancer patients

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Chapter 9
Immunology Breast Cancer

G H. HEPPNER

Although the immunology of human breast cancer has for some time been an area of active investigation there is still little definitive information on such basic questions as the frequency of immune reactions to breast tumor antigens the specificity of those reactions that are detected nor ultimately their clinical significance The reasons for this lack of reliable information are in large measure technical The methodology which has been more or less successfully applied to problems in animal tumor immunology has not yielded the same quality or quantity of data for human cancer where the necessity of working with outbred and in many respects uncontrollable populations is unavoidable As in most other ambiguous situations tumor immunologists tend to react to this dilemma in one of two ways The optimists feel that the data for human cancer are basically in the same direction as that for animals and consequently the conclusions are probably similar The pessimists feel that no amount of sound animal data will ever substitute for weak data in humans and that we might as well admit that we have come no further than BERG (1959) who correlated lymphocytic infiltration with favorable prognosis nearly 20 years ago The purpose of this presentation is to simply review the types of evidence on immune responses in human breast cancer without joining either group In addition experiments suggesting an immunological cross-reactivity between human and mouse mammary cancer will also be discussed

HISTOLOGIC EVIDENCE OF IMMUNE RESPONSES TO HUMAN BREAST CANCER (TABLE 1)

Table 1 Histologic evidence for immune responses to human breast cancer

Observation	Correlation
1 Lymphocytic infiltration of primary tumor	Increased survival [a b c] Low nuclear grade[c]
2 Regional Node	
a Sinus histiocytosis	Increased survival [c d e] Early stage[c]
b Lymphocytic predominance	Increased survival[f]
c Germinal center hyperplasia	Decreased metastasis[f] Fair to poor survival[d f]
d Lymphocyte depletion	Poor survival[f]

[a] BERG (1959)
[b] LANE et al (1961)
[c] BLACK (1970)
BLACK and LEIS (1971b)
[d] HUNTER et al (1975)
[e] DiPAOLA et al (1974)
[f] TSAKRAKLIDES et al (1974)

As mentioned above, there is evidence that either diffuse or perivenous lymphocytic infiltration of primary breast cancers is a prognostically favorable indication (BERG, 1959; LANE et al., 1961; BLACK, 1970) Although controversial, there is increasing agreement that in regional lymph nodes certain changes indicative of an ongoing immune response also have prognostic significance. BLACK (1970) has argued persuasively that the presence of sinus histiocytosis is a favorable sign, a conclusion which has recently been supported by others (DiPAOLA et al., 1974, TSAKRAKLIDES et al., 1974; HUNTER et al., 1975). Further, lymphocyte follicular hyperplasia, without significant germinal center formation, correlates with increased survival (DiPAOLA et al , 1974; TSAKRAKLIDES et al., 1974; HUNTER et al., 1975; BLACK and LEIS, 1971b) whereas regional nodes in which germinal centers are predominant are associated with a survival rate similar to that in patients whose draining nodes appear to be unstimulated (TSAKRAKLIDES et al., 1974) The lowest survival rate is seen in patients whose nodes are depleted of lymphocytes, and show a relative increase in plasma cells (TSAKRAKLIDES et al., 1974

In addition to the ultimate correlation to survival, regional node sinus histiocytosis and follicular hyperplasia are more often seen in patients with in situ than invasive breast carcinoma (BLACK et al , in press) as well as in those without metastasis (TSAKRAKLIDES et al., 1974)1 Furthermore, lymphocytic infiltration of the tumor itself is most often seen in tumors with a low nuclear grade (BLACK et al , in press). Thus, a histologic pattern which is consonant with an active immune response is most often found in patients with the most favorable prognosis. There is also some evidence that in the regional nodes a hyperplastic response in the T cell dependent areas is more favorable than germinal center, or B cell reactivity TSAKRAKLIDES and coworkers have shown that lymph nodes with lymphocyte predominance have a higher percentage of T cells, and lower percentage of B cells, than do nodes with germinal center predominance, although these data were not directly analyzed for prognosis significance (TSAKRAKLIDES et al , 1975).

IMMUNE COMPETENCE IN PATIENTS WITH BREAST CANCER (TABLE 2)

Of great importance to the question of whether patients with breast cancer are making potentially useful immune responses to their cancers is the demonstration that such patients are immunocompetent. In general the immune competence of cancer patients has been most often assayed in regards to cell-mediated immunity, and three types of tests have been employed: (1) skin tests using either recall or primary - dinitrochlorobenzene (DNCB)- antigens, (2) in vitro blastogenic assays of peripheral lymphocytes exposed to "T cell mitogens" such as PHA (phytohemagglutinin) or Con A (Conconavalin A), and (3) counts of total peripheral lymphocytes, or more recently T cells, using the ability of these latter cells to rosette with sheep red blood cells for the assay procedure. Although patients with some types of cancer (lymphomas, sarcomas, and squamous cell carcinomas) do show defects, which moreover correlate with clinical status and prognosis (PILCH et al., 1975) this is in general not so for patients with breast cancer, at least until they are terminal. CATALONA and CHRETIEN (II) found that 26% of patients with all types of adenocarcinoma were anergic, and 27% only marginally sensitized to DNCB, a hapten which is capable of sensitizing over 95% of controls. But the anergy was not related to clinical stage nor was it as frequent as in patients with sarcoma or squamous cell carcinoma. Reactivity to recall antigens has been reported to be impaired (MITCHELL, 1972), somewhat impaired (ROBERTS and JONES-

Table 2 Immune competence in patients with breast cancer

Test	Stage of disease	Observation
Skin test - DNCB	Not specified	26% anergic 27% impaired[a]
Skin test - recall Ags	Localized	Impaired[b]
		Somewhat impaired[c]
		Normal[d]
	Terminal	Depressed[c d]
Blastogenesis to PHA	Primary general	Normal[c d e]
	Terminal	Depressed[c d e]
Effect of sera on blastogenesis	All	Depressed[f]
		No effect[e g]
Total peripheral lymphocytes	All	Normal[d]
Total peripheral T cells	All	Normal[d h]
	After postoperative radiotherapy	Depressed[i]

[a] CATALONA and CHRETIEN (1973)
[b] MITCHELL (1972)
[c] ROBERTS and JONES-WILLIAMS (1974)
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WILLIAMS, 1974) and normal (NEMOTO et al 1974) in breast cancer patients with localized disease but to be grossly deficient in terminally ill patients with wide-spread cancer (ROBERTS and JONES-WILLIAMS 1974; NEMOTO et al 1974) No correlation was seen between skin test reactivity and the presence of lymphocytic infiltration in the tumor or sinus histiocytosis in the regional nodes (MITCHELL 1972; ROBERTS and JONES-WILLIAMS 1974)

Lymphocytes from breast cancer patients were not found to show depressed reactivity in blastogenesis assays except again in advanced disease (ROBERTS and JONES-WILLIAMS 1974; NEMOTO et al 1974; CATALONA et al 1973) Sera from breast cancer patients have been reported able to suppress PHA responsiveness of normal lymphocytes (WHITTAKER et al 1971) but this is not always observed (CATALONA et al 1973) and may be more an effect of doing the assay in homologous rather than autologous sera (WHITEHEAD et al 1974)

The total number of lymphocytes has not been found consistently to fall below normal limits at any stage of breast cancer (NEMOTO et al 1974) nor has the number of peripheral T cells been seen to decline (NEMOTO et al 1974; POTVIN et al 1975) except in patients given postoperative radiotherapy (STJERNSWARD et al 1972)

Thus the general ability of patients with breast cancer to make cell-mediated immune reactions is fairly normal except in advanced disease and does not approach the depression seen in patients with sarcoma or squamous cell carcinoma Humoral immunity is rarely defective in any type of non-lymphoid solid cancer (PILCH et al 1975)

ANTITUMOR ANTIBODY IN PATIENTS WITH BREAST CANCER (TABLE 3)

Several studies aimed at detecting antibody reactive to breast cancer cells in the sera of patients with breast cancer have been reported

As mentioned above, there is evidence that either diffuse or perivenous lymphocytic infiltration of primary breast cancers is a prognostically favorable indication (BERG, 1959; LANE et al., 1961; BLACK, 1970). Although controversial, there is increasing agreement that in regional lymph nodes certain changes indicative of an ongoing immune response also have prognostic significance. BLACK (1970) has argued persuasively that the presence of sinus histiocytosis is a favorable sign, a conclusion which has recently been supported by others (DiPAOLA et al., 1974; TSAKRAKLIDES et al., 1974; HUNTER et al., 1975). Further, lymphocyte follicular hyperplasia, without significant germinal center formation, correlates with increased survival (DiPAOLA et al., 1974; TSAKRAKLIDES et al., 1974; HUNTER et al., 1975; BLACK and LEIS, 1971b) whereas regional nodes in which germinal centers are predominant are associated with a survival rate similar to that in patients whose draining nodes appear to be unstimulated (TSAKRAKLIDES et al., 1974). The lowest survival rate is seen in patients whose nodes are depleted of lymphocytes, and show a relative increase in plasma cells (TSAKRAKLIDES et al., 1974.

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Table 3 Antitumor antibody in patients with breast cancer

Test	Source of sera	Percentage positive sera	Specificity
Indirect immunofluorescence[a]	Breast cancer fibrocystic disease	90%	+ Osteosarcoma, - Other
	Blood bank	0%	
Indirect immunofluorescence[b]	Breast cancer	91%	+ 2/11 breast cancer cultures
	Controls (age, parity, sex-matched)	20%	0/35 others
Immunodiffusion[c] and complement fixation	Breast cancer	46%[d]	+ With other cancers, sarcoma, melanoma
	Fibrocystic disease	34%[d]	
	Fibroadenoma	25%[d]	
	Controls (women/screencees)	1.5%[e]	

[a] PRIORI et al (1971)
[b] EDYNAK et al (1972)
[c] HUMPHREY et al (1974)
[d] At least 1 of 3 samples positive
[e] One sample tested.

PRIORI et al. (1971), using an indirect immunofluorescence test on acetone-fixed cells cultured from breast tumors, observed mainly perinuclear or cytoplasmic fluorescence with sera from 31 of 46 patients with breast carcinoma (42 patients) or fibrocystic disease (4 patients). Only three of these sera, however, reacted with breast cancer cells only, although sera of six patients reacted with cells of fibrocystic disease only, and seven sera reacted with both breast tumor and fibrocytic disease cells. Sera from the other positive patients reacted, in addition to the breast tissue cells, with osteosarcoma cells. Forty-five blood bank sera were negative. Most reactive sera only showed fluorescence with less than 5% of the cultured cells.

EDYNAK et al. (1972) also used indirect immunofluorescence on acetone-fixed cells to detect reactivity in sera from breast cancer patients. Only 2 of 11 breast carcinoma cultures, both derived from pleural effusions, were capable of detecting reactivity, but 22 of 24 sera from breast cancer patients free of metastatic disease 7-10 days after surgery were positive, as compared to 4 of 20 normal sera. The fluorescence was cytoplasmic and seen in 85% of the cells of the positive cultures. Thirty-five additional cultures of normal cells or cells of non breast tumor origin, including seven sarcomas, were negative when tested with sera positive on the two breast carcinoma cultures Unfortunately, these two cultures died out before definitive studies on the specificity of the reactivity and its relationship to clinical status could be carried out.

More recently, HUMPHREY and associates (1974) have reported a breast cancer antigen in "cell sap" of breast carcinoma tissue which reacts by either immuno-diffusion or complement fixation tests, or both, with at least one of three serum samples of 46% breast carcinoma patients, 34% fibrocystic disease patients, 25% fibroadenoma patients, and 1.5% controls (one sample/control only tested). Reactivity is also seen, however, with antigen prepared from a variety of other non breast carcinomas, sarcomas and melanomas. There is some hope that the presence of reactive antibody has prognostic significance since 15 of 18 positive patients with metastatic breast cancer were alive, and disease-free, after 24 months as compared to 2 of 13 negative patients

In addition to these studies which attempted to directly detect anti-breast tumor antibodies there have been numerous reports of the detection in breast cancer patients of serum factors able to interact with cell-mediated immunity This work will be described below

ANTITUMOR CELL-MEDIATED IMMUNITY IN PATIENTS WITH BREAST CANCER IN VIVO STUDIES (TABLE 4)

Table 4 Antitumor cell-mediated immunity in patients with breast cancer In vivo studies

Source of antigen	Stage of patient	Reactivity	Specificity
Cryostat section-[a] autologous tumor	Benign	10% positive	?
	Noninvasive in situ	82% positive	?
	Invasive neg Nodes	47% positive	?
	Invasive pos Nodes	20% positive	?
Solubilized sephadex[b] fraction II polyacrylamide gel electrophoresis fraction			
2a (benign and cancer)	I II	positive	Tissue specific
	IV	negative	
2b (cancer only)	I II	positive	Tumor specific
	IV	positive	

[a] BLACK and LEIS (1973)
[b] HOLLINSHEAD et al (1974)

The pioneers in the in vivo detection of cell-mediated reactivity to breast cancer cells are BLACK and LEIS (1971a b; 1973) Their technique which is the application of a cryostat section of autologous tissue mounted on a skin window coverslip to an abraided area of skin followed in approximately 1 day by evaluation of the nature and amount of cellular infiltration precludes studies of cross-reactivity so that conclusions regarding the specificity and hence immunological nature of their observations cannot be made Nevertheless they have established several interesting points: (1) Cellular infiltrates indicative of hypersensitivity reactions in other skin window procedures are more often induced by cancerous (ca 40%) than by benign breast tissue (10%); (2) Such infiltrates are more often induced by noninvasive precancerous mastopathy and in situ carcinoma (82%) than by invasive carcinoma without nodal involvement (47%) or with nodal metastasis (20%); (3) The detection of positive infiltrates at these stages is consistently seen up to two years postsurgery with the exception of a lower rate of detection during the immediate one month postoperative period; (4) Positive infiltrates are most often seen in patients whose regional nodes taken at the time of mastectomy exhibit positive sinus histiocytosis (see above)

HOLLINSHEAD and associates (1974) are now carrying out a detailed analysis of skin-reactive antigens in solubilized membranes of normal, benign, and cancerous breast cells, as well as of a variety of other tumors. Using Sephadex fractionation of sonicated membranes, followed by polyacrylamide gel electrophoresis, they have identified two skin reactive regions: One is found both in cancer and control breast extracts, and reacted to by breast cancer patients and by patients with other gynecological cancers, but not with several other tumor types; the second reactive band is detected only in extracts of breast cancers and only by breast cancer patients. Interestingly, patients with early stages of breast cancer gave positive skin reactions to both antigens whereas patients with advanced disease reacted only to protein from the second, more specific band.

ANTITUMOR CELL-MEDIATED IMMUNITY IN PATIENTS WITH BREAST CANCER: IN VITRO STUDIES (TABLE 5)

Various in vitro tests for measuring cell-mediated immunity have been applied to breast cancer. ANDERSON et al. (1970) adapted the leukocyte migration inhibition test for this purpose, using a crude homogenate of autologous tissue as antigen. Migration inhibition indices greater for tumor than non-tumor control tissue were seen with leucocytes from 8 of 22 patients with carcinoma and 0 of 9 patients with fibrocystic disease. Leukocytes from control donors were not inhibited. In a similar study, SEGALL et al. (1972) detected inhibition of migration in 8 of 13 breast cancer patients to extracts of autologous tumor, but in only one of six experiments to extracts of breast cancer from other patients, indicating a general lack of cross-reactivity. COCHRAN and associates (1973) using essentially similar techniques, reported cross-reactivity between breast tumors from different patients, but inspection of their data reveals a higher frequency of migration inhibition to extracts of autologous (11 of 14 patients) than of homologous (27 of 59 patients) tumors. Leukocytes from 6 of 19 noncancer control donors were also inhibited, as were leucocytes from 11 of 52 patients with "simple breast conditions" and 2 of 28 with other types of cancer. Extracts from noncancerous breast tissue inhibited leukocytes from 5 of 26 breast cancer patients.

A subsequent paper of COCHRAN et al (1974) also claims cross-reactivity between breast cancers on the basis, this time, of nearly identical frequencies of inhibition to autologous (56%) as to homologous (53%) tumor extracts. A problem with their analysis, however, is that their control group is heavily weighed (96 of 157) with individuals who have simple breast conditions and who, as a group, shows a lower percentage of reactivity than do normal donors. Whatever this may mean, it would seem to exclude them from the category of "control". Doing so, one is left with nearly 30% of normal control donors who react against breast cancer extracts. Since leukocytes from the normal donors are, of course, only tested against homologous extracts, this high rate of normal reactivity makes it very difficult to evaluate whether or not reactivity of breast cancer patient leukocytes to homologous breast cancer extracts indicates a cross-reactive, breast tumor antigen. As will be seen, a similar difficulty applies to data obtained with the microcytotoxicity assay.

Inhibition of leukocyte migration against breast cancer extracts seems to show minimal correlation with clinical stage, although patients with visceral metastases show less frequent reactivity than do those with axillary node, or no node involvement (COCHRAN et al., 1974).

Table 5 Antitumor cell-mediated immunity in patients with breast cancer in vitro studies

Assay	Percentage Positive Reactions				
	Autologous patients	Homologous patients	Benign patients	Controls	Clinical correlation
Leucocyte migration inhibition					
1) extracts	36%[a]	-	0%	0%	-
	60%[b]	17%	-	0%	None
	79%[c]	46%	21%	31%	-
	56%[c]	53%	16%	27%	Visceral metastases ↓
2) sections in situ	90%[d]	36%	0%	4%	Sinus histiocytosis Stage
invasive					
- nodes	46%	15%			
6+ nodes	24%				
Leukocyte adherence inhibition	85% (origin not specified)		0%	6%	Visceral metastasis ↓
Colony inhibition and microcytotoxicity					
1) CMI	~90%[f h]	50-95%[f h]	17%[g], ~100%[i]	Normal: 25-93%[g h]	None
2) SBF	-	-	-	Other cancer - ?	Metastases ↑

a ANDERSON et al (1970)
b SEGALL et al (1972)
c COCHRAN et al (1973, 1974, 1972)
d BLACK et al (1974a b)
e GROSSER and THOMSON (1975)

f HELLSTRÖM et al (1971a; 1971c)
g FOSSATI et al (1972)
h HEPPNER (1973) HEPPNER et al (1975)
i AVIS et al (1974)

CMI: cell-mediated immuniy
SBF serum blocking factor

In contrast to cell-mediated immunity, which in the main is detectable by microcytotoxicity assay in nearly all breast cancer patients regardless of clinical status (HELLSTRÖM et al., 1971a; HEPPNER, 1973) serum blocking factors are most often found in patients with metastatic disease (HEPPNER, 1973; HELLSTRÖM et al. 1971c). The Hellströms have also reported a factor in sera from four breast cancer patients who were clinically tumor-free which could "unblock" blocking sera, allowing anti-tumor cell-mediated immunity to function in vitro (HELLSTRÖM et al., 1971b). They have described one clinically-free breast cancer patient whose sera could both "arm" lymphocytes from control donors or potentiate lymphocytes from breast cancer patients to kill breast cancer target cells (HELLSTRÖM et al., 1973).

One general comment about the in vitro testing for cell-mediated immunity to breast cancer cells seems in order. Most workers have used breast cancer cells, or extracts thereof, from patients with invasive disease. For microcytotoxicity testing cultures started from metastatic pleural effusions are frequently used because they are relatively easy to cultivate. Black's data, however, (see above) suggest that invasive carcinomas are not only less antigenic than local disease, they are less likely to have cross-reactive antigens. Clearly much more attention will have to be given to the stage of the breast cancer used as antigen for in vitro testing.

IMMUNOLOGICAL CROSS-REACTIVITY BETWEEN HUMAN AND MURINE BREAST CANCER (TABLE 6)

One other area of human breast tumor immunology which has received much attention is the possible immunological cross-activity between human and mouse breast cancer. The mouse tumors to which the comparison has been made are those causally associated with MTV (mammary tumor virus), so that cross-reactivity with human breast cancer is suggestive evidence of a similar viral etiology. Antibodies capable of reacting with MTV or with mouse breast tumors have been detected in the sera of women with breast cancer by a number of techniques, including viral neutralization (CHARNEY and MOORE, 1971), fixed immunofluorescence (DMOCHOWSKI, 1973), and peroxidase-labelling (HOSHINO and DMOCHOWSKI, 1973). The relationship of the antibodies detected to human breast cancer remains, however, problematical since only few and inadequate control sera have as yet been tested and the frequency

Table 6 Immunological cross-reactivity between human and murine breast cancer

Reactivity detected	Assay	Relationship to disease
Antibody to MMTV or mouse tumor cells	Neutralization[a] Immunofluorescence[b] Peroxidase-Ab[c]	? Too few samples tested
Cell-mediated immunity to MMTV-positive milk	Leukocyte[d] Migration inhibition	Correlate with other indicators of CMI Correlates with stage of disease

[a] CHARNEY and MOORE (1971)
[b] DMOCHOWSKI and PRIORI (1973)
[c] HOSHINO and DMOCHOWSKI (1973)
[d] BLACK et al (1974, 1975).

of detection with breast cancer patient sera is low Also human sera have been reported as reacting with either type B (HOSHINO and DMOCHOWSKI 1973) or type A (MULLER et al 1973) viral particles

The strongest evidence for an immunologic relationship between human and mouse breast cancer is the work of BLACK et al (1974a b; 1975) who have shown that migration of the leukocytes of about 30% of breast cancer patients can be inhibited by mouse milk containing MTV or purified MTV but not by virus-free milk The pattern of reactivity is similar to that seen to cryostat sections of breast cancer (see above) in that reactivity is more often seen with leukocytes from patients with in situ than with invasive disease Also reactivity to virus-positive milk is correlated in individual patients with reactivity to breast cancer tissues Interestingly leukocytes from patients less than 45 years old react to MTV-containing milk much less frequently (5%) than do leukocytes from older patients (32-39%) BLACK and associates (1975) have also shown a positive correlation between the presence of sinus histiocytosis and other favorable histologic signs and reactivity to MTV although leukocytes from patients negative for the histological signs showed a higher frequency of reactions to virus than to autologous cancer tissues Positive correlation was also seen between reactivity to MTV and skin window hypersensitivity tests (see above)

More recently BLACK and coworkers (in press) have identified in eluates of cryostat sections proteins found only in immunogenic breast cancer tissues which band similarly to protein component(s) of MTV in polyacrylamide gel electrophoresis This component may also correspond to the distinctive band of HOLLINSHEAD et al (1974) It would seem that another molecular probe for viral activity in human cancers may soon be identified

CONCLUSIONS

The evidence for immune reactivity to human breast cancers has been reviewed and is summarized in Tables 1-6 It is evident that much of the data are weak and inconclusive even in regard to such a basic question as the specifictiy of the immune response or of the antigen detected In my opinion the most meaningful work has been done by BLACK and associates who started their study by correlating histological reactions with clinical status then added an in vivo hypersensitivity test then in vitro methodology and only recently turned to tumor extracts and viral probes They have been careful however to always relate each new discovery back to the beginning - the clinical and pathological findings This sequence of study assures that whatever is found will be clinically relevant It is especially important that all Black s studies have stressed the basic difference in immunogenicity between in situ and invasive breast cancer since nearly all other workers have ignored this distinction This difference in immunogenicity coupled with clear evidence that breast cancer patients are in general immunocompetent regardless of clinical stage has clear implications for treatment and especially for immunotherapy

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Chapter 10
Potent Inhibitory Activity of a New Antiestrogen, RU 16 117, on the Development and Growth of DMBA Induced Rat Mammary Adenocarcinoma

F LABRIE P A. KELLY J ASSELIN and J P RAYNAUD

INTRODUCTION

Mammary carcinoma induced in the rat by dimethyl-benzanthracene (DMBA) administration (HUGGINS et al 1961) is well-known to be dependent upon estrogens and prolactin (MEITES 1972; QUADRI et al 1974; CASSELL et al 1971; LEUNG et al 1975; DAO 1962) for its development and growth

In fact procedures which reduce estrogen activity (ovariectomy anti-estrogens) or which reduce circulating levels of prolactin (hypophysectomy ergot drugs) have been shown to reduce the number and size of these tumors (PEARSON et al 1969; DAO 1962; MEITES 1972 NAGASAWA and YANAI 1970; TERENIUS 1968; CASSELL et al 1971; QUADRI et al 1974; HEUSON et al 1971) Moreover tumor growth can be reinitiated in ovariectomized animals by treatment with estrogens or prolactin (MEITES 1972; TALWALKER et al 1964; LEUNG et al 1975)

Since we have recently found that RU 16117 (11α-methoxyethinyl estradiol) has more potent antiestrogenic properties than the compounds previously available (FERLAND et al 1975; RAYNAUD et al 1975) it seemed of interest to study its effect on the development and growth of DMBA-induced mammary tumors in the rat and to compare the effect of this treatment with the beneficial effect following castration

Moreover since measurements of hormone receptor levels in tumor tissue can be of great help in predicting the hormone dependency of human breast cancer (JENSEN et al 1975; McGUIRE et al 1975; HORWITZ et al 1975) the levels of receptors for estradiol-17β progesterone and prolactin were correlated with the response to hormonal treatment

MATERIALS AND METHODS

Treatments

Female Sprague-Dawley rats (obtained from Canadian Breeding Farms St-Constant Québec) were administered 20 mg DMBA in 1 ml corn oil by gastric gavage at 50-55 days of age Animals were housed 2 per cage with a lighting regimen of 14 hr light- 10 hr-darkness (lights on between 5:00 and 19:00 hr) and received Purina rat chow and water ad lib

In the experiment on the effect of RU 16117 on tumor development the animals were injected subcutaneously daily from the day DMBA (Sigma) was administered with RU 16117 at doses of 0 5 2 8 or 24 μg or with

the vehicle alone (0.1 ml 1% gelatine-0.9% NaCl). RU 16117 was first dissolved in a small volume of ethanol before further dilution with the vehicle. A group of animals were ovariectomized (OVX) the day following DMBA treatment. At the outset of the experiment, there were 18-20 animals per group

For the study of the effect of RU 16117 on tumor growth, animals with palpable tumors were selected approximately four months after administration of DMBA Tumor number and size were then recorded for each rat at weekly intervals for three weeks before beginning treatment.

Animals were then divided into groups of 13-14 rats so that the outset of treatment, each group had approximately the same average number and size of tumors. The animals were treated daily for 4 weeks with 0.1, 0.5, 2.5, or 12 5 µg estradiol-17ß (E_2), or 2, 8, or 24 µg RU 16117 injected in 0.1 ml 1% gelatin-0.9% NaCl, or the vehicle alone. For comparison, a group of animals were ovariectomized.

At weekly intervals, animals were examined for mammary tumors by palpation and the number of tumors per rat was recorded The two largest perpendicular diameters of each tumor were measured with calipers and the product of these diameters was used as estimate of tumor size Animals were sacrificed by decapitation between 08:00 and 09:00 h and trunk blood was collected in heparinized beakers. Following separation by centrifugation, plasma was stored at -20° C until hormone assays.

Preparation of Cytosol and Membrane Fractions

After decapitation of the animals, the mammary tumors were removed, freed of connective and adipose tissue, and rinsed in ice-cold buffer B (10 mM Tris-HCL, pH 7.4, 1 5 mM EDTA and 10 mM thioglycerol). The tumors were then weighed and homogenized in 3 volumes (w/v) of buffer A (25 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 10 mM thiglycerol and 10% glycerol) using a Polytron PT-10 homogenizer at a setting of 5 for 2 periods of 10 s with an interval of 10 s for cooling. The homogenate was centrifuged at 18,000 xg for 15 min and the resulting supernatant was then centrifuged at 105,000 xg for 90 min to obtain the cytosol (supernatant) fraction. All steroid binding assays were performed with fresh cytosol. The 105,000 xg pellet was resuspended in 25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ with a Teflon-glass homogenizer and stored at -20° C until peptide binding assays Protein concentration was measured (LOWRY et al., 1951) using bovine serum albumin as standard.

[^{3}H] R 5020 and [^{3}H] Estradiol-17ß Binding Assays

The potent synthetic progestin R 5020 (17, 21-dimethyl-19-nor-pregna-4, 9-diene-3,20-dione) was used for measurements of progesterone receptor levels (ASSELIN et al , 1976). The use of this compound avoids the difficulties met with [^{3}H] progesterone which binds to corticosteroid-binding globulin and has a relatively fast dissociation rate from its receptor.

[^{3}H] R 5020 and [^{3}H] estradiol-17ß binding were measured using the dextran-coated charcoal assay as described (ASSELIN et al , 1976).

Binding Assays of ^{125}I-Labeled Ov ... Bovine GH and Porcine Insulin

Specific binding ... ined i ... brane fractions as previ-
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protein in a final volume of 0 5 ml in 25 mM Tris-HCl 10 mM $MgCl_2$ (pH 7 4) containing 0 1% bovine serum albumin The incubation was performed in duplicate at 25° C in the presence or absence of an excess of unlabeled hormone (1 µg) for 60 min (insulin) 3 h (GH) or 6h (PRL) The incubation was stopped by the addition of 3 ml of the incubation buffer Bound and free hormone was separated by low speed centrifugation (2000 rpm) at 4° C in an IEC PR 6000 centrifuge for 25 min

Radioimmunoassays

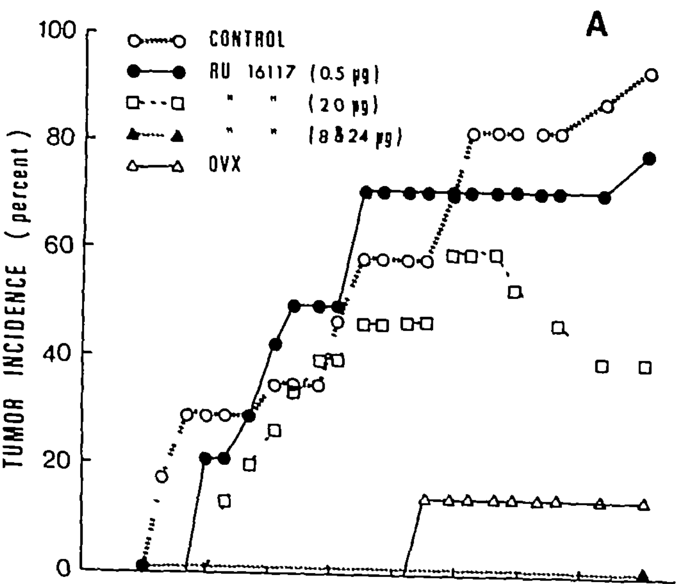
Plasma LH and PRL were measured by double-antibody radioimmunoassays (BIRGE et al 1967; ODELL et al 1967) using rat hormones (LH-I-3 LH-RP-1 PRL-I-1 and PRL-RP-1) and rabbit antisera (anti-rat LH serum I and anti-rat PRL-S-2) kindly provided by Dr A F Parlow for the National Institute of Arthritis and Metabolic Diseases Rat Pituitary Hormone Program Radioimmunoassay data were analysed with a desk-top Hewlett-Packard calculator using a program written in this laboratory and based on model II of RODBARD and LEWALD (1970) All data are expressed as mean ± SEM Statistical significance was calculated according to the multiple-range test of DUNCAN-KRAMER (1956)

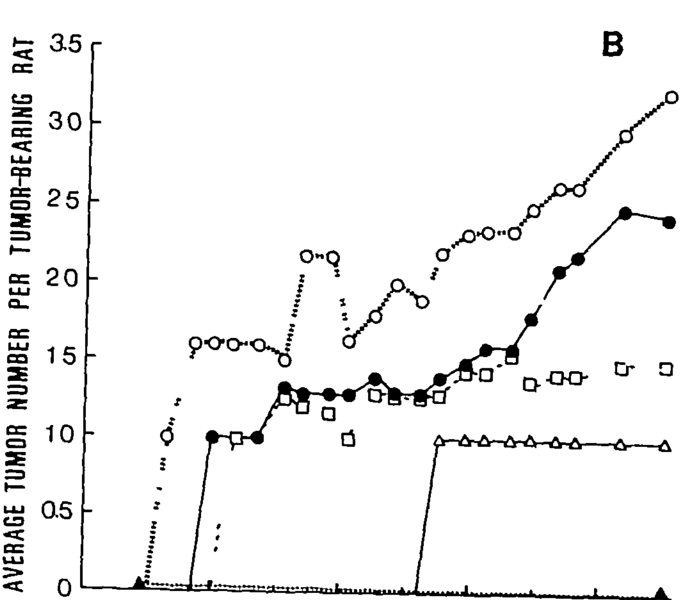
RESULTS

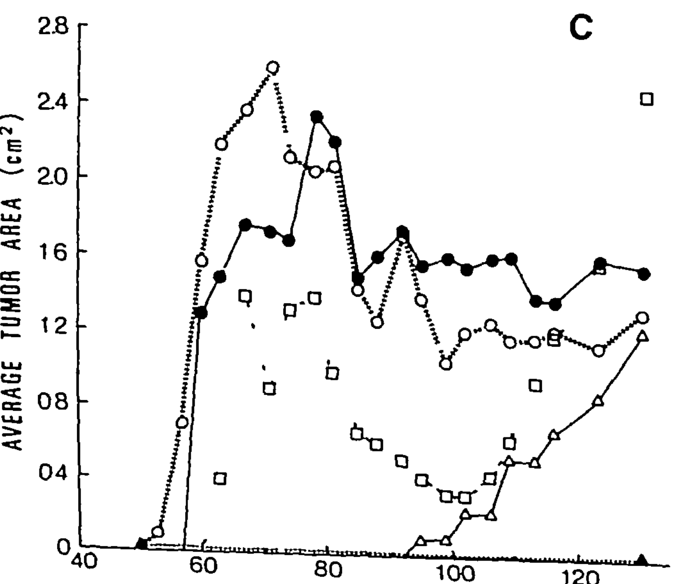
Effect of RU 16117 on Tumor Development

As illustrated in Figure 1A all treatments resulted in a delayed onset of tumor appearance After a delay of 10 days RU 16117 at a dose of 0 5 µg per day resulted in a curve similar to that of controls, although the incidence reached only 78 6% When RU 16117 was injected at a dose of 2 µg per day the maximal incidence was 60% from 99-106 days after which the incidence fell to a value of 40% at day 130 The important finding is however that RU 16117 at doses of either 8 or 24 µg per day completely inhibited tumor development in all animals Ovariectomy completely inhibited tumor appearance until day 95 when 2 out of 14 animals developed palpable tumors (14 2%)

The average number of tumors per tumor-bearing animal is shown in Figure 1B Once again control animals showed a gradual increment reaching a maximum of 3 25 tumors per rat 130 days after DMBA administration At doses of 0 5 and 2 µg per day RU 16117 resulted in a reduction to 2 50 and 1 50 tumors per animal respectively As mentioned earlier only one tumor developed in each of the two ovariectomized rats These two tumors became however quite large (Chart 1C) In control rats the largest tumor size was 2 61 cm^2 on day 71 after which the values declined to approximately 1 2 cm^2 for the remainder of the study Treatment with 0 5 µg RU 16117 had no significant effect on tumor size while rats receiving 2 µg RU 16117 showed a more complex pattern of tumor growth Initially tumor size increased to approximately 1 2 cm^2 after which there was a diminution in size to an average tumor area of only 0 35 cm^2 on day 102 with a progessive increase to a value of 2 51 cm^2 on day 130 It should be mentioned however that this increase in average tumor area between days 102 and 130 in the group treated with 2 0 µg RU 16117 is due to only one large tumor which accounted for 80% of the total tumor area of this group at the end of the experiment In the absence of this tumor average tumor area would have continued to decline in this group to reach a value of 0 42 cm^2 on day 130







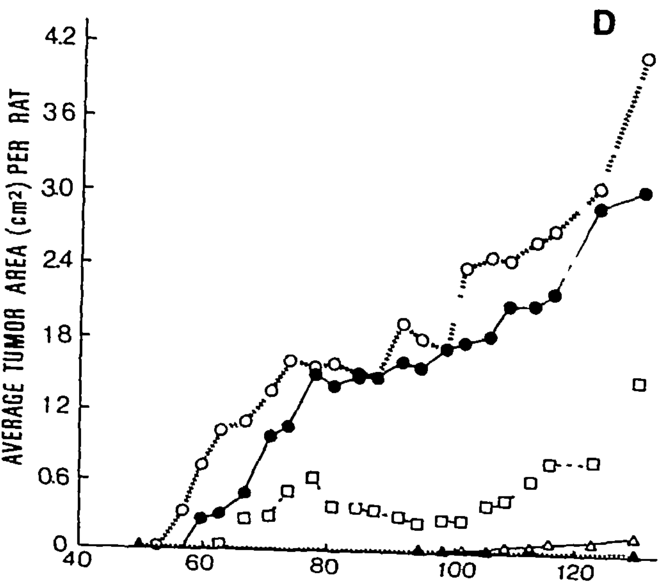


Fig 1 Effect of treatment with increasing doses of RU 61117, or ovariectomy (OVX) on the development of DMBA-induced mammary tumors Injections began the day DMBA was administered and continued for the next 130 days Ovariectomy was performed the day following DMBA administration. Animals were examined twice weekly for the presence of tumors and when present, tumor area (length x width) was measured (A) Tumor incidence as a function of time after DMBA. (B) Average number of tumors per tumor-bearing animal. (C) Average tumor area (cm2) (D) Average tumor area (cm2) per animal

Specific binding of [^{3}H] estradiol [^{3}H] R 5020 and ^{125}I-ovine PRL to DMBA-induced mammary tumors is shown in Figures 2A B and C respectively Binding of [^{3}H] E_2 was 5 1 ± 1 0 pmoles/g tissue in tumors from control animals while after daily treatment with 0 5 and 2 0 μg RU 16117 reductions of binding to 2 7 ± 0 3 and 1 9 ± 0 6 pmoles/g tissue were observed Binding of [^{3}H] R 5020 was 8 9 ± 1 5 pmoles/g tissue in the control group Daily injection of 2 μg of the steroid resulted in a reduction to 4 5 ± 0 5 pmoles/g tissue In one tumor which developed in the ovariectomized group the level of progesterone receptors was very low at 0 6 pmoles/g tissue RU 16117 treatment caused also a reduction of the binding of ^{125}I-PRL to tumor plasma membranes from 6 3 ± 1 5% in control rats to 2 9 ± 0 6% in tumors from rats receiving 2 μg RU 16117 per day (Fig 2C) In the one tumor from an ovariectomized rat there was very low binding of ^{125}I-oPRL (1 2%) Specific binding of both ^{125}I-insulin and ^{125}I-bovine growth hormone was constant at 0 2 to 0 4% in all treatment groups (data not shown)

Since DMBA-induced mammary tumors are PRL-dependent (CASSELL et al , 1971; LEUNG et al 1975; MEITES 1972; NAGASAWA and YANAI, 1970; PEARSON et al 1969; KELLY et al 1974) it was of interest to examine the effect of RU 16117 treatment on plasma PRL levels The doses of 2 0 and 8 0 μg of the steroid caused a nonsignificant decrease of plasma PRL levels while the highest dose (24 μg) increased the levels from 21 ± 7 to 41 ± 10 ng/ml ($p<0\ 05$) (data not shown) Ovariectomy decreased the plasma PRL concentration to 2 0 ± 0 3 ng/ml ($p\ 0\ 01$)

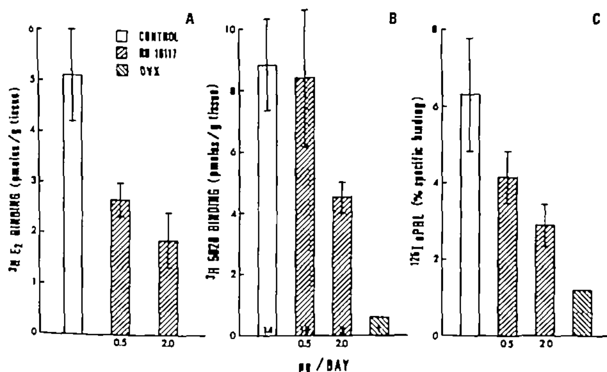


Fig 2 Specific binding of [^{3}H] estradiol-17β (A) [^{3}H] R 5020 (B) and ^{125}I-oPRL (C) to DMBA-induced rat mammary tumors from rats treated with increasing doses of RU 16117 or ovariectomized Rats were given a single injection of DMBA and treatment with RU 16117 commenced the same day All tumors with an area larger than 1 cm^{2} on the day animals were sacrificed (day 130) were removed Cytosol and particulate membrane fractions were prepared and specific hormone binding was determined as described under Materials and Methods

As illustrated in Figure 3, treatment with RU 16117 led to a progressive decrease of plasma LH levels, the effect becoming significant at the 8 µg ($p<0.05$) and 24 µg ($p<0.01$) doses. Plasma FSH concentration (not shown) were not affected by the antiestrogen treatment whereas, ovariectomy led, as expected, to a marked increase of both LH and FSH plasma levels.

As illustrated in Figure 4A, while 4-week treatment with 2 µg of RU 16117 has little effect on the growth of DMBA-induced mammary tumors, doses of 8 and 24 µg lead to 45% and 65% inhibitions of tumor number respectively. In the control animals, a linear increase from 3.23 ± 0.55 to 4.54 ± 0.75 tumors/rat was observed during the 4 weeks of treatment. It can also be seen that ovariectomy, a treatment well known to cause tumor regression (*HUGGINS, 1965; QUADRI et al , 1974*) has an effect very similar to that of a daily dose of 24 µg RU 16117. At the high dose, RU 16117 not only markedly decreased the number of tumors, but it also led to a marked reduction of the total tumor size (Fig 4B) Lower doses of the antiestrogen had little or no effect on total tumor area.

In order to ascertain that the inhibitory effect of RU 16117 on tumor growth was not due to the low estrogenic activity of the compound (FERLAND et al., 1975; RAYNAUD et al., 1975), the effect of increasing doses of E_2 was examined under the same experimental conditions. As shown in Figure 5, daily injection of 0.1, 0.5, 2.5, or 12,5 µg E_2 has no significant effect on the number of tumors. For easier comparison, values for control and ovariectomized animals have been included again

While the total area was decreased from 4.0 ± 0.4 to 0.6 ± 0.2 cm^2/rat after castration, the two low doses of E_2 induced somewhat larger tumors while the two larger doses resulted in similar or slighter smaller tumor size (Fig. 5B). When only tumors present at the beginning of treatment are considered, 0.5 µg E_2 is found to have a stimulatory effect on tumor size while the other doses have no significant effect.

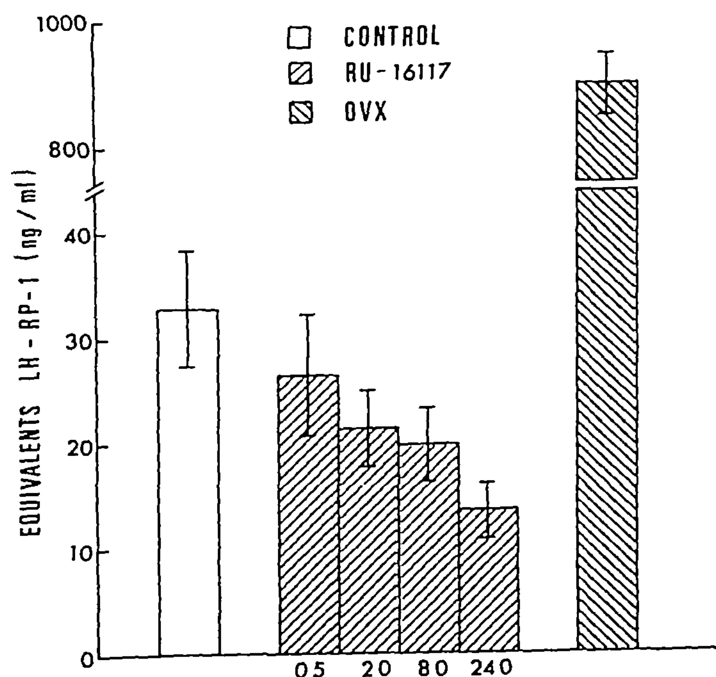


Fig 3 Effect of RU 16117 or ovariectomy on plasma LH levels in rats injected with DMBA. Conditions were as described in Figure 1

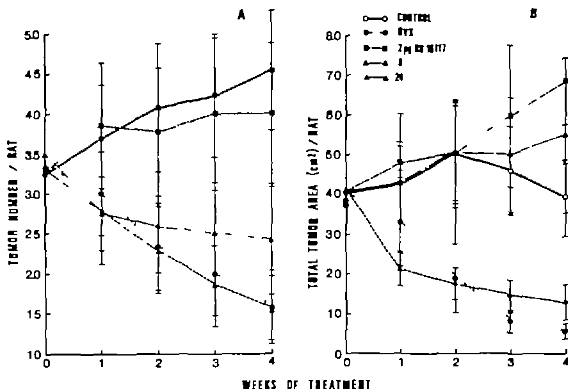


Fig 4 Effect of 4-week treatment with 2 8 or 24 µg of RU 16117 daily or ovariectomy on the number of DMBA-induced mammary tumors per rat (A) and total tumor area per animal (B) Treatment was started approximately 4 months after DMBA administration

When the effect of 4-week treatment with E_2 RU 16117 or ovariectomy was examined on specific binding of [^{3}H] E_2 [^{3}H] R 5020 and ^{125}I-oPRL binding of [^{3}H] E_2 was 2 3 ± 0 4 pmoles/g tissue in tumors from control animals while after daily treatment with 24 µg RU 16117 this value was lowered to 0 3 ± 0 1 pmoles (data not shown) The slight decrease of [^{3}H] E_2 binding in the groups treated with 2 or 8 µg RU 16117 was not significant Except for the dose of 0 1 µg E_2 which resulted in an increased level of estrogen receptors E_2 was without significant effect on the concentration of E_2 receptors in tumor tissue

The level of progestin receptors was not significantly affected at any of the doses of E_2 used While the two lower doses of RU 16117 were without effect the 24 µg dose was also apparently ineffective However if 2 tumors having high values of 16 0 and 14 8 pmoles/g tissue were eliminated from the group the level of [^{3}H] R 5020 receptor would be reduced to 2 6 ± 1 5 pmoles/g tissue after treatment with 24 µg RU 16117 The level of this receptor was reduced to 0 1 ± 0 1 pmole/g tissue after ovariectomy

Binding of ^{125}I-oPRL to the particulate membrane fraction was only significantly reduced by treatment of rats with 24 µg RU 16117 for the most part closely paralleling the results of [^{3}H] E_2 binding Ovariectomy was however without effect on PRL binding Binding of ^{125}I-insulin and ^{125}I-hGH remained constant in all tumors examined the binding ranging from 0 3 to 0 4 ± 0 1% and 0 3 to 0 6 ± 0 1% respectively (data not shown)

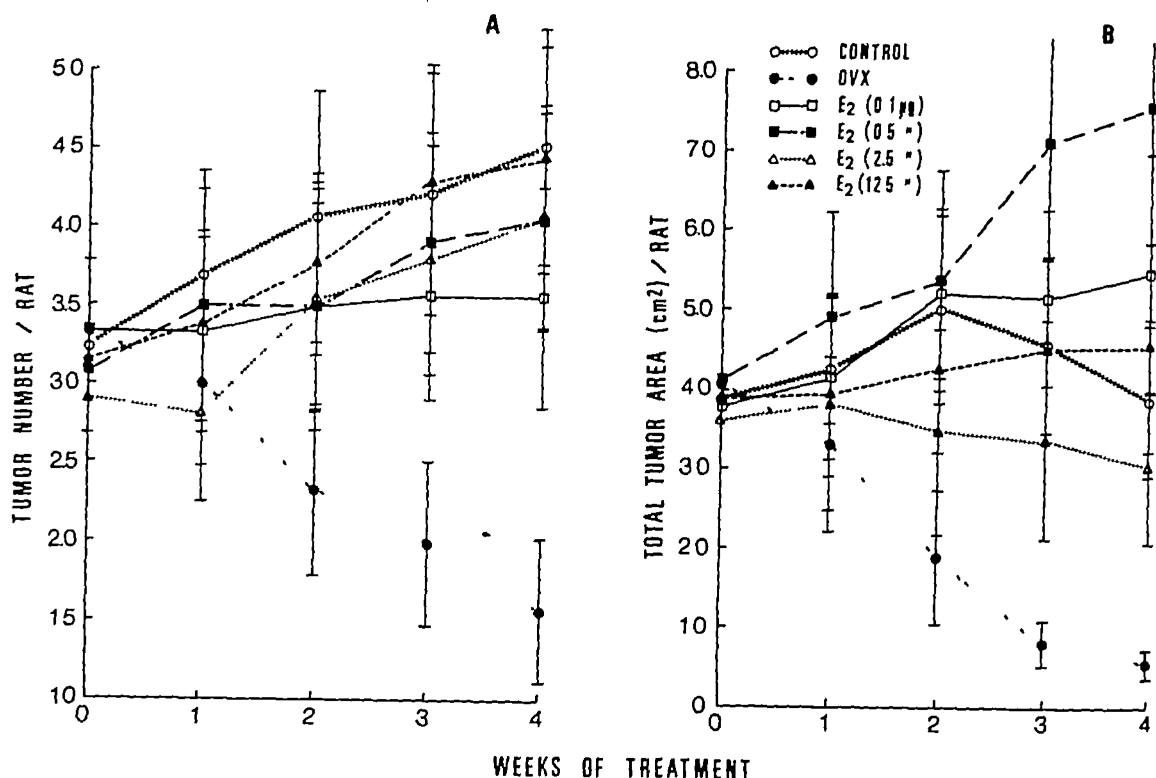


Fig 5 Effect of 4-week treatment with 0.1, 0.5, 2.5 or 12.5 µg estradiol-17ß or ovariectomy or the number of DMBA-induced mammary tumors per rat (A) and total tumor area per animal (B) Treatment was started approximately 4 months after DMBA administration

DISCUSSION

The present data show that the new antiestrogenic compound RU 16117, at the relatively low doses of 8 and 24 µg/day, is capable of completely preventing the appearance of mammary tumors after DMBA administration This compound has weak estrogenic activity in the mouse (Rubin test = 1/100 of estradiol-17ß) and castrated (Allen DOISEY test = 1/20 of estradiol-17ß) and competes for uterine estradiol cytosol receptor in mouse (1/20 of estradiol-17ß) and rat (1/10 or estradiol-17ß) RAYNAUD et al., 1975). When injected at low doses (1 or 2 µg) on estrus, RU 16117 has been shown to: (1) inhibit the spontaneous LH and FSH peaks on the afternoon of proestrus, (2) inhibit the 7-10-fold increase of pituitary sensitivity to LH-RH usually seen on afternoon of proestrus, (3) block ovulation, and (4) delay the appearance of vaginal cornification (FERLAND et al., 1975; FERLAND et al., unpublished observations.

While the two highest doses of RU 16117 led to complete inhibition of tumor development, the two lower doses had also a significant inhibitory effect. The net effect is best illustrated in Figure 1D where it can be seen that treatment with 0.5 or 2.0 µg RU 16117 leads to inhibition of average tumor size to respectively 75% and 40% of control. This important effect results from lower incidence and smaller tumors after treatment with low doses of the antiestrogen.

As well illustrated in Figures 4 and 5, RU 16117 can not only block tumor development but, at the daily dose of 24 µg, it can also cause tumor regression in rats with already established mammary tumors. In

fact after 4 weeks of treatment the average number of tumors per rat and the tumor size are reduced to approximately 30% of control

It is well known that estrogens can have a dual effect on mammary carcinoma induced by DMBA Injection of estrogens stimulates tumor growth in ovariectomized animals (LEUNG et al 1975) while in animals with already developed tumors large doses of estrogens can lead to tumor regression (HUGGINS 1965) or inhibition of its development (MEITES et al 1971; KLEDZIK et al 1974) At a dose of 20 μg/day estradiol benzoate has been found to be effective in preventing growth of established DMBA-induced mammary tumors (MEITES et al 1971) That the observed inhibitory effect of RU 16117 is not due to the low estrogenic activity of the compound is clearly indicated by the absence of inhibitory effect of similar treatment with a range of doses of E_2 (0 1-12 5 μg/day) which cover the estrogenic activity of the doses of RU 16117 used

Although requiring much higher doses other antiestrogens have been found to inhibit DMBA-induced tumor development and growth As examples CI-128 at the dose of 1 mg/kg was found to be somewhat less efficient than ovariectomy in inhibiting DMBA-induced mammary tumor growth (DE SOMBRE and ARBOGAST 1974) At the daily dose of 5 mg given for 40 days starting 20 days before DMBA administration MER-25 reduced tumor incidence to only 40% of control (KLEDZIK et al 1974) When given every other day at the dose of 1 mg/kg for 30 days in rats which had developed tumors nafoxidine led to an important inhibition of tumor growth (TERENIUS 1971) while in another study the same drug at the daily dose of 1 mg/kg started 2 weeks after DMBA almost completely prevented tumor development measured after 6 or 12 weeks (HEUSON et al 1971)

In human breast cancer it now appears quite clear that a good correlation exists between the level of estrogen receptors and the response of this cancer to hormonal therapy (JENSEN et al 1975; McGUIRE et al 1975) It has also been suggested that the simultaneous presence of receptors for estradiol and progesterone in the tumor tissue increases the predictive value of receptor measurements (HORWITZ et al 1975) In DMBA-induced tumors a correlation has been found between estradiol receptor levels and hormonal dependency of the tumors (MOBBS 1966; *TERENIUS 1968; McGUIRE and JULIAN 1971) Moreover* a good correlation has been obtained between the level of receptors for PRL and the response of these tumors to stimulation of growth by PRL (KELLY et al 1974)

The present data clearly show that treatment with 2 μg RU 16117 led to a 40-60% reduction of the levels of receptors for estradiol progesterone and PRL in the mammary tumors Levels of progesterone and PRL receptors were reduced to 10-15% of control after ovariectomy a finding confirmed on a larger scale in subsequent experiments (ASSELIN et al unpublished observations)

In the study on the effect of treatment on the growth of already established mammary tumor decreased levels of receptors for E_2 PRL and progesterone were found in the tumors from ovariectomized animals while the dose of RU 16117 (24 μg) efficient to inhibit tumor growth had a similar inhibitory effect on the levels of E_2 and PRL receptors

These findings of low levels of hormone receptors after ovariectomy or treatment with 24 μg RU 16117 may indicate that tumors unresponsive to hormonal treatment are those with low levels of receptors or that ovariectomy or RU 16117 treatment cause a reduction of receptor levels A possible mechanism of action of RU 16117 in the tumor tissue could

be a decrease of the hormone receptor level leading to relative unresponsiveness of the tissue to its hormonal environment.

Since we have found that RU 16117 inhibits LH and FSH secretion at all doses used and treatment with the 24 μg dose inhibits tumor growth in the presence of increased plasma prolactin levels, it thus appears likely that the potent inhibitory activity of RU 16117 on the development and growth of DMBA-induced mammary carcinoma results from actions at both the hypothalamo-pituitary and tumor levels, the action at the tumor level being possibly exerted through modification of steroid and prolactin receptor concentrations.

SUMMARY

When initiated the same day as dimethylbenzanthracene (DMBA) administration, daily treatment with 8 or 24 μg of the new antiestrogen RU 16117 (11α-methoxy ethinyl estradiol) completely prevented the appearance of mammary tumors in all animals up to the last time interval studied (130 days after DMBA administration). At daily doses of 0.5 and 2.0 μg of RU 16117, the tumor incidence was reduced to 78.6% and 40.0%, respectively. The levels of receptors for estradiol, progesterone, and prolactin in tumor tissue were reduced after treatment with 2.0 μg RU 16117 while the binding of growth hormone and insulin was not affected. While plasma LH levels were decreased after treatment with 8 or 24 μg RU 16117, plasma prolactin levels were slightly increased in animals receiving the highest dose of the antiestrogen.

When RU 16117 was given at the daily dose of 24 μg for a period of 4 weeks, RU 16117 led to 65% reduction of the number of already established DMBA-induced mammary tumors. Not only the tumor number but also the tumor size was reduced by RU 16117 in a manner similar to that following ovariectomy. That the inhibitory effect of RU 16117 was not due to its low estrogenic activity is indicated by the absence of inhibitory effect of similar treatment with a range of doses (0.1-12.5 μg per day) of estradiol-17β which cover the low estrogenic activity of the doses of RU 16117 used. Decreased levels of receptors for estradiol-17β, progesterone, and prolactin were found in the tumors remaining after ovariectomy while treatment with the dose (24 μg) of RU 16117, efficient to inhibit tumor growth, has a similar inhibitory effect on the levels of estradiol-17β and prolactin receptors.

The present data indicate that the potent inhibitory effect of RU 16117 on the development and growth of DMBA-induced mammary tumors results from actions at both the hypothalamic-pituitary and tumor levels. The action at the peripheral level would be possibly secondary to a reduced sensitivity of the tissue to circulating hormones through lowering of hormone receptor concentrations.

Abbreviations

The abbreviations used are: RU 16117, 11α-methoxy-19-nor-17α-1,3,5 (10)-pregnatrien-20-yne-3,17-diol, DMBA,7,12-dimethylbenz(a)anthracene; GH, growth hormone; PRL, prolactin; CI-628, 1-[2-p-[α(p-methoxy phenyl) -β-nitrostyryl phenoxy ethyl pyrrolidine; MER-25, (1-[p-(2-diethylaminoethoxyphenyl]-2(p-methoxyphenyl)-1-phenylethanol); Nafoxidine, U-11100A, 1- 2-[p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphtyl) phenoxy ethyl]-pyrrolidine hydrochloride.

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Chapter 11
Steroid Receptor Proteins and Regulation of Growth in Mammary Tumors

N BRUCHOVSKY and E. VAN DOORN

INTRODUCTION

Since the action of different steroidal hormones is expressed only in cells containing specific hormonal receptors it is entirely reasonable to suppose that the presence of receptors per se might serve as a useful marker of hormonal responsiveness To be sure if the receptor is indispensable for the survival of the cell the acute withdrawal of hormone from a receptor-containing cell should be sufficient cause for the cell to die Unfortunately according to the results on estrogen receptors in human breast cancer compiled from the separate investigations of 13 groups from eight countries (McGUIRE et al 1975) and summarized in Table 1 this expectation has not been wholly fulfilled

Table 1 Estrogen receptors in human breast cancer Condensed from the report of McGUIRE et al (1975)

Therapy	Response	
	ER+	ER-
Ablative	59/107 = 55 %	8/94 = 8%
Additive	51/85 = 60%	7/82 = 8%

When the estrogen receptor (ER) test is positive only 55-60% of the tumors regress after ablative or additive endocrine therapy Since 40-45% of the tumors which contain estrogen receptor fail to respond it is clear that the receptor mechanism is not an accurate marker of hormonal responsiveness Despite this practical shortcoming one should not overlook the observation that when the estrogen receptor test is negative just 8% of the tumors regress after ablative or additive endocrine therapy Therefore in the absence of estrogen receptors the response rate is lower as might be expected but certainly the result would be more consistent with theory if the response rate were zero in these tumors Surprisingly the observation suggests that a tumor may remain responsive even if estrogen receptor is not present

In focussing on the more important discrepancy we suggest that there are three major reasons for the lack of response in estrogen receptor positive cancers as summarized in Table 2 First the criteria of response may be too strict Tumors which regress slightly or in some other way do not conform to the definition of response will be overlooked Second the response may not be totally mediated by the cytoplasmic receptor detected by the assay Third the genome may be unable

Table 2. Reasons for lack of response in ER+ cancers

1	Criteria of response too strict.
2.	Response is not totally mediated by cytoplasmic receptor
3	Genome unable to respond to regulatory molecules (cytoplasmic receptor, nuclear receptor, steroid)

to respond to regulatory molecules. The latter would include the cytoplasmic receptor, nuclear receptor, and steroid. A fourth possibility is that a certain number of tumors are comprised of hormone-sensitive and insensitive cell populations, but in the absence of any clear-cut evidence for such cellular divergence in human mammary cancers (STOLL, 1970) this prospect will not be amplified in the present review. For a comprehensive description of the complexity of hormonal responses in human breast cancer, the reader is referred to the excellent series of articles by STOLL (1970; 1972; 1973; 1974).

Since the importance of the receptor mechanism in hormone-sensitive cells is unquestionably preempted by the importance of the hormonal responses capable of being elicited in such cells, the first part of our review is devoted to an account of the fundamental effects of a steroid hormone as observed in an experimental model system.

BASIC RESPONSES OF A HORMONE-SENSITIVE ORGAN

For reasons of practical facility our studies on hormonal responsiveness have involved the extensive use of two animal models, namely the rat ventral prostate and the androgen-dependent Shionogi mouse mammary carcinoma (BRUCHOVSKY et al., 1975a; BRUCHOVSKY and LESSER, 1976). The triphasic response of the prostate to the administration of androgens is depicted in Figure 1. When the number of cells in the gland is below normal, DNA synthesis and cell proliferation are initiated by androgen therapy; the number of cells increases until the size of the gland is restored to normal, and then cell proliferation stops abruptly. The size of the gland is maintained as long as hormone is present, but upon its withdrawal, the gland regresses and the number of cells is reduced to the basal level. The three phases of response have been termed initiation, negative feedback, and autophagia, and it has been postulated that each phase is controlled by a separate gene - an initiator, nullifier, and autophage, respectively (BRUCHOVSKY et al., 1975a; BRUCHOVSKY and LESSER, 1976).

THEORETICAL BASIC RESPONSES OF NEOPLASMS

Knowledge of factors which control negative feedback will unquestionably be an essential element in the future understanding of neoplastic change in hormone-sensitive tissues. The deletion of negative feedback, with or without further deletion of initiation and autophagia, in theory would have predictable consequences on the growth patterns of cells as shown in Figure 2. In the absence of the negative feedback effect (Fig. 2A), growth remains dependent on the supply of hormone, but the number of cells increases indefinitely owing to the lack of negative feedback control. However, withdrawal of hormone induces an autophagic response and regression of tissue is observed. This type

Fig 1 Basic responses of hormone-sensitive organ Groups of 3-7 rats castrated 7 days previously were treated with daily doses of 400 μg of dihydrotestosterone /100 g body weight and at various times number of cells /prostate was determined by measuring number of nuclei in glandular tissue as described by LESSER and BRUCHOVSKY (1973) Shaded area under curve indicates period of hormonal treatment Withdrawal of hormone as by castration is followed by reduction in number of cells/prostate to the basal level Three basic responses are initiation of DNA synthesis and cell proliferation negative feedback and autophagia (BRUCHOVSKY and LESSER 1976)

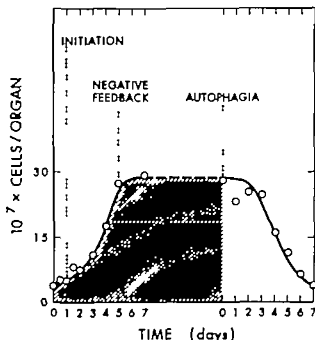
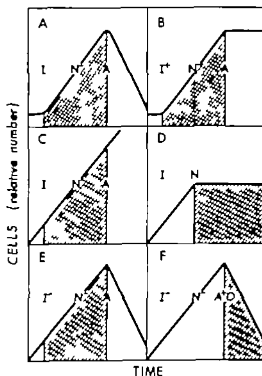


Fig 2 Theoretical basic responses of neoplasms Deletion of negative feedback (N) with or without further deletion of initiation (I) and autophagia (A) in theory would have predictable consequences on growth patterns of cells as shown Plus and minus symbols denote presence and absence respectively of a given response $A^{+}0$ indicates that normally restricted pathway to autophagic response is unblocked (open)



of growth curve best describes the response of the DMBA induced rat mammary tumor (McGUIRE and JULIAN 1971; TERENIUS 1974; BOYLAN and WITTLIFF 1975; LEUNG and SASAKI 1975) of the transplantable rat mammary tumor MTW9 (COSTLOW et al 1975) and of certain human breast cancers (LEE and SPRATT 1972; BURN 1974; HEUSON 1974)

In the absence of both negative feedback and autophagia (Fig 2B) initiation of growth remains under hormonal control but when hormone is withdrawn growth simply stops and the tissue mass remains more or less constant in size For the most part this type of response is characteristic of the Shionogi mouse mammary carcinoma (BRUCHOVSKY and LESSER 1976)

The further absence of initiation (Fig. 2C) renders the tissue completely unresponsive; hormone has no effect on growth because the phases of initiation, negative feedback, and autophagia are not expressed.

At least two more growth patterns can be predicted, depending on whether the tissue retains the capacity to express negative feedback (Fig. 2D) or autophagia (Fig. 2E) in the absence of the expression of the other two constraint mechanisms. As will be described later such responses are fairly common in patients undergoing treatment for breast cancer.

A final growth pattern whose occurrence has been documented clinically (STOLL, 1970; LEE and SPRATT, 1972; HEUSON, 1974) is depicted in Figure 2F. On the basis of the mechanisms described in Figure 1, the type of response in which autophagia is induced by the administration of hormone would not be predicted. However, more detailed examination of control of the autophagic response indicates that hormone not only endows the cell with the capacity to undergo such a response, but also prevents expression of this capacity if the hormone is present in excess (BRUCHOVSKY et al., 1975a; BRUCHOVSKY and LESSER, 1976). Therefore in some cases it seems that only the first part of this mechanism is functional leaving the pathway to an autophagic response completely open.

CLINICAL OBSERVATIONS ON THE RESPONSES OF NEOPLASMS

An extensive commentary on clinical experience in the management of breast cancer and other hormone sensitive cancers is beyond the scope of this review, but very complete information is available from several authoritative sources (HAYWARD, 1970; STOLL, 1970, 1972, 1973, 1974; BURN, 1974; HEUSON, 1974). The fundamental responses of breast cancer can be classified into three categories as presented in Table 3 The first is cell proliferation which is often stimulated by low doses of estrogen and inhibited by high doses. Not only are such effects observed in vivo but also in vitro in tissue cultures of breast cancer (LIPPMAN and BOLAN, 1975). The second response is regression of tumors, induced by ablative therapy in premenopausal women, and paradoxically by additive therapy in postmenopausal women, an opposite treatment Under certain conditions both forms of therapy may be effective in either age group (STOLL, 1970, 1972; BURN, 1974; HEUSON, 1974). The third category is rebound regression, an objective remission after discontinuation of therapy (HEUSON, 1974).

Table 3. Clinical responses of breast cancer

1. Cell proliferation

   Low doses of estrogen stimulate growth
   High doses of estrogen inhibit growth

2. Induced regression

   Ablative (or additive) therapy in premonopausal women
   Additive (or ablative) therapy in postmenopausal women

3 Rebound regression

   Objective remission after discontinuation of therapy

Even this brief outline of evidence based on clinical observations is sufficient to confirm that fluctuations in hormone levels can produce changes in the physiological state of human breast cancer similar to those predicted in Figure 2

COORDINATION OF BASIC RESPONSES

In order to explain the coordination of responses that occur in a hormone-sensitive organ a scheme of interrelated plus and minus controls such as the one shown in Figure 3 is ultimately required (BRUCHOVSKY et al 1975a; BRUCHOVSKY and LESSER 1976) The initiator produces a plus signal which is responsible for switching on DNA synthesis and cell proliferation in the presence of an adequate concentration of hormone The nullifier produces a minus signal which is responsible for switching off DNA synthesis and cell proliferation when the tissue reaches a normal size and accounts for negative feedback The autophage produces a plus signal which programs a cell for its eventual destruction by capacitating the autophagic mechanism However this process is inhibited by hormone which provides a minus signal A fall in the concentration of hormone below critical levels required for the maintenance of a differentiated cell thus stimulates autolysis and removal of cells The expression of all three genes is thought to take place sequentially as defined by a temporal order during progression of cells through the cell cycle (VAN DOORN et al 1976)

The vertical bars suggest that the initiator autophage and nullifier are activated or potentiated when hormone concentrations reach specific

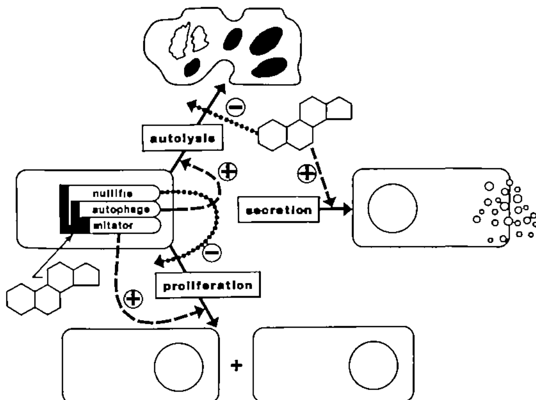


Fig 3 Coordination of basic responses by initiator nullifier and autophage genes (BRUCHOVSKY and LESSER 1976)

levels during sensitive periods of the cell cycle. In addition to affecting these particular genes and inhibiting cellular autolysis, hormone promotes secretory activity by stimulating transcriptional or post-transcriptional processes (ICHII et al., 1974; LIANG and LIAO, 1975; BULLER and O'MALLEY, 1976).

THEORETICAL CAUSES OF UNRESPONSIVENESS

However useful the foregoing deductions on the nature of hormonal responses and their control, our insight into these vital mechanisms can be further amplified through examination of an opposite condition of the cell, namely the condition of unresponsiveness. In very general terms the potential causes of unresponsiveness are summarized schematically in Figure 4. As suggested previously the normal cell (Fig. 4A) contains three genes, the initiator, nullifier, and autophage which control responsiveness of cells. Hormone is transported from the cytoplasm into the nucleus, where fluctuations in the concentration of hormone regulate genetic activity. Implicit in this hypothesis is that hormone interacts with the genome although the interaction may not be as direct as implied by the graphic. Signal generation unquestionably involves several intermediate steps, and the requirement for hormone receptor is virtually certain (BULLER and O'MALLEY, 1976). Theoretically, a cell remains responsive as long as one of the three genes is functional and remains accessible to hormone. In the example shown in Figure 4B the nullifier is rendered inactive by an inferred change in the conformation of chromatin. In the first type of unresponsiveness (Fig. 4C) resistance to hormone develops as a result of the failure of hormone to penetrate the nucleus. Certain of the genes may be functional but remain dormant because no activating signals are generated in the absence of nuclear hormone. In the second type of unresponsiveness (Fig. 4D) the cell transports hormone into the nucleus but the

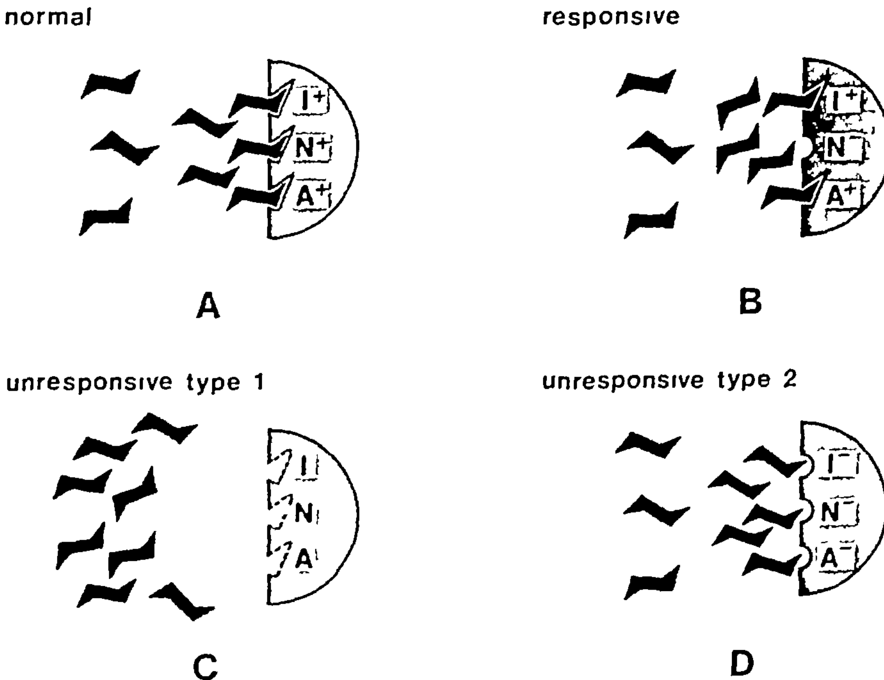


Fig. 4. Theoretical causes of unresponsiveness

interaction of hormone with nuclear components is abnormal and no regulatory signals develop

From this consideration of the unresponsive condition one can appreciate that there are probably two important factors which control the degree of sensitivity of a cell to steroidal hormones The first is the mechanism that regulates the concentration of hormone in the nucleus and the second is the mechanism through which binding of hormone in the nucleus is achieved Further pursuit of information about these mechanisms inevitably forces one to examine the subject of steroid-hormone receptors

POSSIBLE MECHANISMS OF STEROID-HORMONE ACTION

In a hormone-sensitive gland such as prostate androgenic hormones are capable of stimulating new rounds of cell division with attendant synthesis of hormonal receptors (VAN DOORN et al 1976; Fig 10) This process is depicted in Figure 5A On the basis of indirect evidence it may be inferred that receptor is synthesized following the transport of hormone into the nucleus where through direct effects on chromatin the hormone induces transcription with resultant production of messenger RNA coding for receptor (BRUCHOVSKY et al 1975b 1975c; BRUCHOVSKY and CRAVEN 1975) An alternative possibility yet to be ruled out is that hormone induces the synthesis of receptor by acting primarily on translation In either case the newly synthesized receptor together with hormone is thought to be translocated into the nucleus during which process it undergoes a structural modification The latter change renders the receptor molecule larger or smaller depending on the system being studied (KING and MAINWARING 1974) As long as hormone levels in the nucleus remain high the receptor is restricted to the nuclear site Acute withdrawal of hormone from the cell appears to cause the efflux of receptor from the nucleus and the reversion of receptor to a

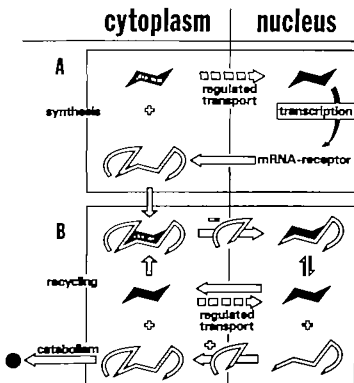


Fig 5 Possible mechanisms of steroid-hormone action A) proliferating cell B) nonproliferating differentiated cell

basic configuration (VAN DOORN et al., 1976; Fig. 11) as depicted in Figure 5B. The cytoplasmic receptor so formed is presumably recycled if hormone levels return to normal. However, if subnormal levels of hormone persist the receptor is catabolized and the cell is destroyed by autophagic processes.

Whether the recycling of receptor actually takes place remains a moot point since the equivalence of cytoplasmic and nuclear receptors has not been established irrefutably in any experimental model system. It remains possible that transport of free hormone takes place independently and that binding to cytoplasmic receptor only facilitates this reaction, or alternatively that binding is merely a phenotypic marker of hormonal action closely linked to steroidal transport into the nucleus.

As shown in Figure 6 the nucleus would normally be expected to acquire three ligands each of which can potentially bind to DNA. An increasing body of evidence suggests that the hormone-receptor complex is an important regulatory molecule capable of stimulating transcription and indirectly translation (DAVIES and GRIFFITHS, 1975; BULLER and O'MALLEY, 1976; KALIMI et al., 1976). For the response to go to completion, it is clear that the binding sites on DNA, whether defined in terms of acceptor sites or effector sites (BULLER and O'MALLEY, 1976) would have to be functional.

In summary, it is conceivable that cellular responses are regulated indirectly by cytoplasmic receptor which ostensibly might serve as a mechanism to expedite the transport of hormones into the nucleus, or regulated directly by nuclear receptor whose effects are presumably exerted through its binding to DNA We will next outline a series of experiments in which both possibilities were examined.

PARTIAL ISOLATION OF STEROID-HORMONE RECEPTORS

In order to measure the concentration of androgen receptors during different phases of responsiveness of the prostate, both in vivo and in vitro procedures were used to label cytoplasmic and nuclear binding proteins with radioactive androgen. A full description of these methods is given in previous publications (BRUCHOVSKY et al , 1975b; BRUCHOVSKY and CRAVEN, 1975) and details are provided in the legends to Figures 7-11.

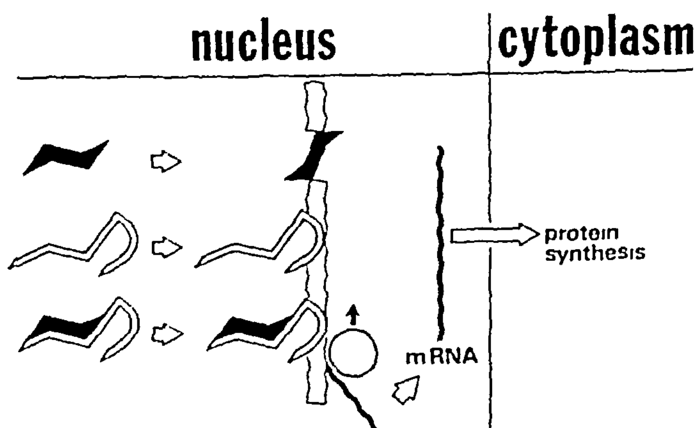


Fig. 6 Interactions between ligands and DNA

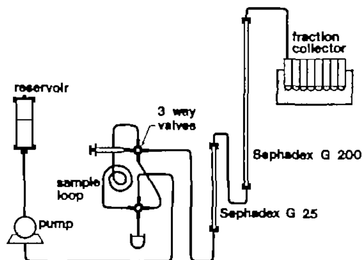


Fig 7 Sephadex G-25 G-200 dual-column chromatography

Partial isolation of androgen-receptor complex is accomplished by gel-exclusion chromatography using a dual column system shown in Figure 7 The sample containing labelled receptors is drawn into a sample loop which is then switched on-line by means of three-way valves The sample is then pumped through a short column of Sephadex G-25 and then through a long column of Sephadex G-200 Fractions of 1-2 ml are collected and analyzed for radioactivity

In the resulting chromatogram shown in Figure 8 nuclear androgen-receptor complex is recovered in fractions 40-50 and is characterized by a sedimentation coefficient of 3 3 S in 600 mM NaCl(Fig 8 insert)

By comparison cytoplasmic androgen-receptor complex is recovered in fractions 30-45 of the corresponding chromatogram shown in Figure 9 and is characterized by a sedimentation coefficient of 4 4 S in 600 mM NaCl (Fig 9 insert)

EFFECT OF ORCHIECTOMY ON THE CONCENTRATION OF ANDROGENS AND OF ANDROGEN RECEPTORS IN THE PROSTATE

With this approach the amount of androgen and of androgen receptor in prostate have been measured at two different times following orchiectomy (BRUCHOVSKY and CRAVEN 1975) As shown in Table 4 one day after orchiectomy 70 000 androgen molecules can be transferred into the nucleus and our most accurate estimate indicates that about 9 000 are bound to receptor In the same tissue at the same time there are only 8 000 cytoplasmic receptor molecules/cell A similar discrepancy appears seven days after orchiectomy when the nucleus can be stimulated to take up 22 000 androgen molecules and to form 4000 nuclear receptors when there are fewer than 1000 cytoplasmic receptor molecules/cell Thus in both cases the quantity of cytoplasmic receptor is insufficient to account for the total influx of androgens into the nucleus if a mole to relationship is assumed It is clear that the presence of cytoplasmic receptor is associated with an enhanced ability of the cell to incorporate androgens into the nucleus However the final concentration of androgens in the nucleus may be regulated in part by a mechanism which does not rely on the translocation of cytoplasmic receptor

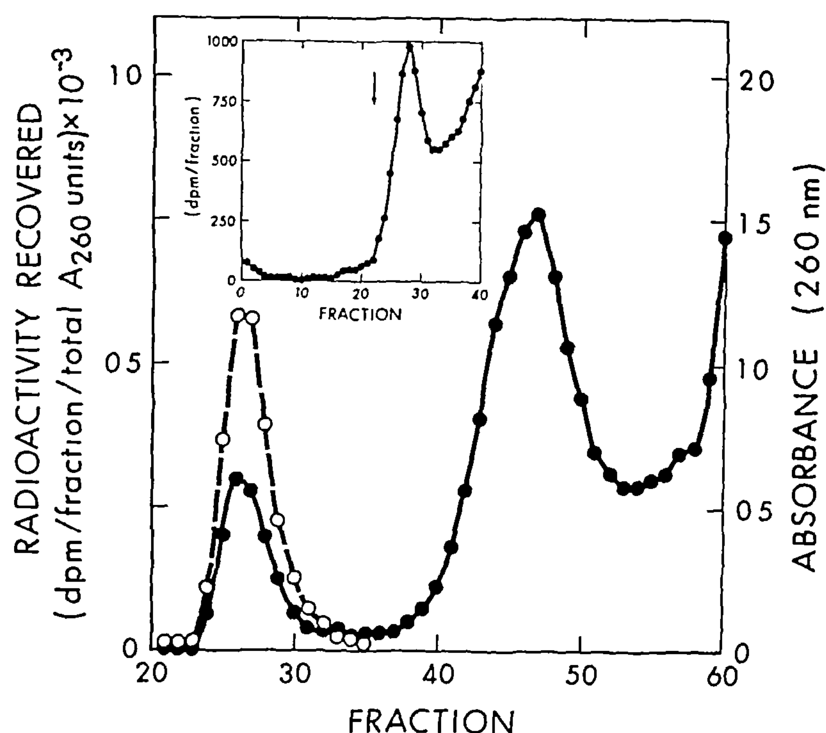


Fig. 8 Partial isolation of nuclear receptor Purified nuclei were disrupted by sonication and extracted with Tris/EDTA buffer, pH 7.0, containing 600 mM NaCl (BRUCHOVSKY et al , 1975b) The extract was incubated for 18 h at 20° C in presence of |1, 2-^{3}H| dihydrotestosterone (40 Ci/mmole) at conc of 20 nM After incubation, sample was divided into two equal parts One part was analyzed for binding by Sephadex G-25 G-200 dual-column chromatography The other was treated with dextran-coated charcoal, concentrated, and analyzed for binding by sucrose density-gradient centrifugation (insert) Column effluent was collected in fractions of 1 2 ml Absorbance measured at 260 nm, O Radioactivity recovered, ● Amount of radioactivity in receptor peak was calculated by doubling area under most clearly defined half-portion of peak Arrow shown in insert marks position of hemoglobin sedimentation standard (From VAN DOORN et al., 1976)

RECEPTOR FLUX IN THE REGENERATING PROSTATE

Since the responsiveness of cells seems to be affected quite profoundly by the nuclear concentration of hormone, we have suggested that cytoplasmic receptor could influence responsiveness indirectly by altering the level of hormone in the nucleus. Another way in which the regulation of responsiveness might be achieved is through a direct effect of hormone receptors on growth controlling mechanisms This premise was tested by measuring the concentration of cytoplasmic and nuclear receptors during active and quiescent phases of prostatic growth.

Beginning 7 days after orchiectomy rats were treated with daily injections of dihydrotestosterone to stimulate DNA synthesis and cell proliferation and restore the prostate to its normal size. The receptor flux over a period of growth spanning 14 days was followed, and the results are presented in Figure 10A. Within 12 h of the first injection, the number of molecules of nuclear receptor (open circles) increases from 0-5000/cell and continues to increase to a maximum of

Table 4 Effect of orchiectomy on concentration of androgen and androgen receptors

Days after orchiectomy	Site	Number of molecules	
		Androgen	Receptor
1	Nucleus	70 000	9000
	Cytoplasm		8000
7	Nucleus	22 000	4000
	Cytoplasm		<< 1000

Groups of 3-14 rats (250-300 g) orchiectomized 1 and 7 days previously received i v injections of 300μ Ci (6 9 nmoles) of [1 2-^{3}H] testosterone After 60 min the animals were killed and the radioactivity in cytoplasmic and nuclear fractions of prostate was measured and calculated in terms of number of molecules of androgen in each fraction

Cytoplasmic receptor was isolated as follows Prostatic tissue (1 g) was minced and incubated with [1 2-^{3}H] dihydrotestosterone (750 nM) for 2 min at 37°C The tissue was extensively washed and then separated into cytosol and nuclear fractions by centrifugation techniques (BRUCHOVSKY et al 1975b) The cytosol fraction was treated with ammonium sulfate at 80% saturation and the protein precipitate was analyzed by Sephadex G-25:G-200 dual-column chromatography (BRUCHOVSKY et al 1975b) Radioactivity in the eluted receptor peak was summed and the number of molecules of receptor was calculated assuming that 1 molecule of androgen is equivalent to 1 molecule of receptor
Nuclear receptor was isolated as follows Prostatic tissue (1 g) was minced and fractionated as before into cytoplasmic and nuclear samples Purified nuclei were disrupted by sonication and extracted with Tris/EDTA buffer pH 7 0 containing 600 mM NaCl (BRUCHOVSKY et al 1975b) The extract was then incubated in the presence of [1 2-^{3}H] dihydrotestosterone (40 Ci/mmole) at a concentration of 20 nM for 18 h at 20° C Analysis of the extract was then accomplished by Sephadex G-25: G-200 dual-column chromatography and the amount of radioactivity in the receptor peak was determined by summation Finally the number of molecules of receptor was calculated assuming that 1 molecule of androgen is equivalent to 1 molecule of receptor

12 000/cell at 2 days This level is maintained for the next 3 days and then falls to 9000/ cell by the 7th day of treatment There is a further gradual decline to about 8000/cell over the remaining period of 7 days

During the same time course of tissue regeneration the apparent number of cytoplasmic receptors (closed circles) remains constant at 1000/cell It should be stressed that the latter result is probably a gross overestimate of the actual number of cytoplasmic receptors owing to our method of calculating the size of the receptor peak (VAN DOORN et al 1976)

From the results in Figure 10A it is clear that the flux in the level of nuclear receptor is not in phase with the changes in rate of DNA synthesis (broken line) DNA synthesis is initiated 12 h after the number of nuclear receptors at 9 000/cell is almost at a maximum More significantly the negative feedback effect on DNA synthesis manifested between the 3rd and 4th days of treatment occurs in the presence of a constant level of nuclear receptor These two observations indicate that any effect of nuclear receptor on DNA synthesis must be indirect and that other factors besides nuclear receptor are probably responsible for switching DNA synthesis on and off

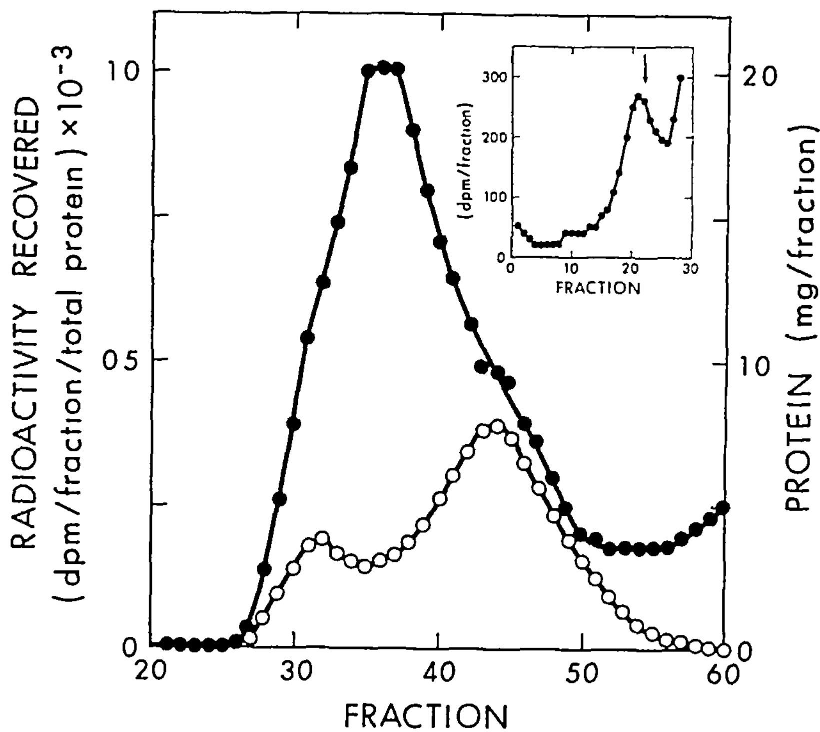


Fig 9 Partial isolation of cytoplasmic receptor Prostatic tissue was obtained from rats orchiectomized 24 h previously, minced and incubated for 2 min at 37° C in presence of |1, 2-^{3}H| dihydrotestosterone at conc of 750 nM Cytosol fraction was treated with ammonium sulfate at 80% saturation, and the protein precipitate was analyzed for binding by Sephadex G-25 G-200 dual-column chromatography and by sucrose density-gradient centrifugation (insert) Column effluent was collected in fractions of 1 4 ml Amount of radioactivity in the receptor peak was calculated as described in the legend to Figure 2 Recovery of protein, O, radioactivity, ● Arrow shown in insert marks position of hemoglobin sedimentation standard (From VAN DOORN et al., 1976)

The dependence of de novo receptor synthesis on cell proliferation is well demonstrated by the results shown in Figure 10B. The product of the number of receptors/nucleus as taken from Figure 10A, and the number of cells/prostate as taken from Figure 10B (broken line), yields the number of nuclear receptors/prostate and is indicated by the open circles in Figure 10B. Comparison of the upper and lower curves in Figure 10B reveals a distinct parallelism between the number of nuclear receptors/prostate and the number of cells/prostate On the 5th day of treatment when cell proliferation is curtailed by the negative feedback effect, there is no further net synthesis of nuclear receptor. It is to be noted that the decrease in the number of nuclear receptors/cell between the 5th and 14th days of treatment is offset by a slight increase in the number of cells/prostate allowing the number of nuclear receptors/prostate to remain constant.

Perhaps the most unexpected feature of this da its fa e-
provide evidence of any coherence between th
ceptor and the period of cell differentiati
days of treatment when protein synthesis i
tion by androgens (LESSER and BRUCHOVSKY,

A final point that merits emphasis is tha
responsive to hormone when the level of c
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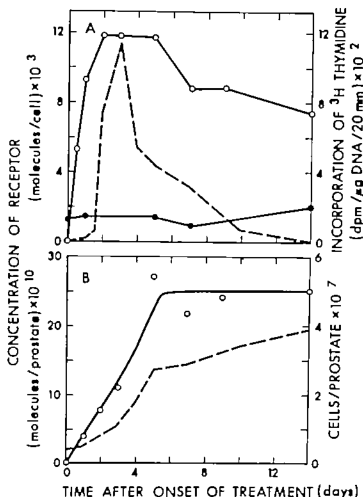


Fig 10 Receptor flux in regenerating prostate Groups of 3-12 rats orchiectomized 7 days previously were treated with daily s c injections of dihydrotestosterone (400 µg/100 g body weight) Prostatic tissue was recovered at the times shown and analysed for presence of cytoplasmic and nuclear receptors by methods described in legends to Figures 7 9 A) concentration of nuclear receptor O and of cytoplasmic receptor ● expressed as molecules cell B) concentration of nuclear receptor expressed as molecules/prostate Broken line in A) indicates rate of DNA synthesis measured in terms of incorporation of ^{3}H-thymidine and in B) indicates number of cells in regenerating prostate as replotted from data of LESSER and BRUCHOVSKY (1973) (From VAN DOORN et al 1976)

RECEPTOR FLUX IN THE INVOLUTING PROSTATE

One of the first noticeable effects of lowering the concentration of androgens in the adult male animal as by orchiectomy is a rapid decline in the basal rate of DNA synthesis to 25% of normal within 24 h (LESSER and BRUCHOVSKY 1973) In contrast to the rapid effects on DNA synthesis both prostatic weight and the number of cells in the prostate do not drop significantly below the normal level until the 4th day after orchiectomy (Fig 11 broken line) and then decline to about 30% and 15% of normal by the 7th day

As shown by the results in Figure 11 (open circles) these changes are accompanied by a decrease in the number of nuclear receptors from a starting level of 7000/cell to a level of 500/cell within 24 h None

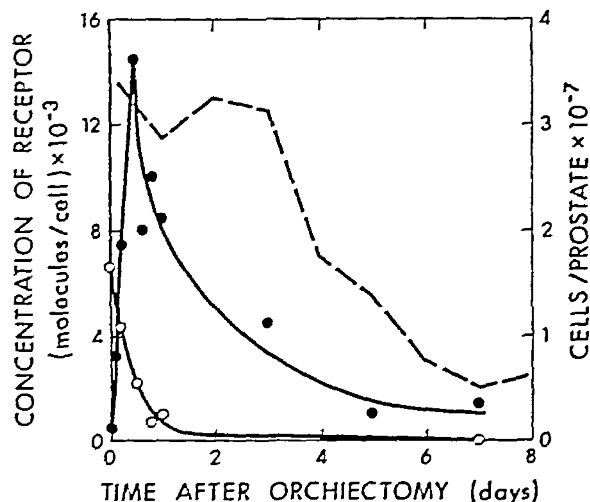


Fig 11 Receptor flux in involuting prostate Prostatic tissue was recovered from orchiectomized rats at intervals following surgery and analyzed for presence of cytoplasmic and nuclear receptors as described in the legends to Figures 7-9. Concentration of nuclear receptor, O, and of cytoplasmic receptor, ●, expressed as molecules/cell Broken line indicates number of cells in involuting prostate as replotted from data of LESSER and BRUCHOVSKY (1973) (From VAN DOORN et al., 1976)

are detected at 7 days. Initially, there is an opposite change in the number of cytoplasmic receptors (closed circles) which increases from less than 1000/cell in the normal animal to a maximum of 14,000/cell at 16 h after orchiectomy. Following this upward change, the number of cytoplasmic receptors falls rapidly reaching the threshold level of 1000/cell by the 5th day. Since the regression of prostate is preceded by a marked reduction in the number of both nuclear and cytoplasmic receptors, it may be inferred that receptor catabolism and autophagia are related phenomena.

INCORPORATION OF ANDROGENS AND THE RESPONSIVENESS OF SHIONOGI MOUSE MAMMARY CARCINOMAS

The subject material presented in the foregoing parts of this review illustrates how a normal tissue responds to hormone and to a certain degree it furnishes insight into the relationship between hormone receptors and responsiveness. We will now examine the question whether similar relationships can be observed in neoplastic cells as exemplified by the Shionogi mouse mammary carcinoma. The sources of two responsive lines and seven unresponsive lines used in the following experiments are listed in Table 5.

Investigations were initially carried out to determine whether any differences could be detected in the ability of responsive and unresponsive lines to incorporate androgens. The effect of increasing doses of radioactive testosterone ranging from 10 µCi (0.23 nmoles) to 300 µCi (6.9 nmoles) on the amount of androgen incorporated into whole tumor and tumor nuclei during a 30 min period was studied exhaustively with dependent line 1 and autonomous lines 1 and 3 (BRUCHOVSKY et al., 1975c). It was found that the incorporation of

Table 5 Sources of tumor lines

Tumor line	Code	Source
Responsive		
1	SC-115	Toronto
2	SC-115	Fukushima-ku
Unresponsive		
1	SC-115-A1	Edmonton
2	Ka3	Toronto
3	SC-140	Toronto
4	SC-42	Fukushima-ku
5	Kc3	Toronto
6	CIV	Toronto
7	AIV	Toronto

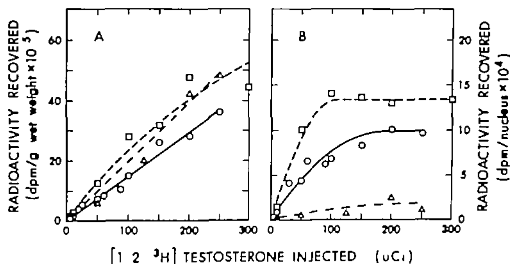


Fig 12 Incorporation of androgens and responsiveness of Shionogi mouse mammary carcinomas Male mice with tumors of dependent line 1 and female mice with tumors of autonomous lines 1 and 3 were injected i v with increasing doses of [1 2-³H] testosterone ranging from 10 µCi (0 83 nmoles) to 300 µCi (8 9 nmoles) and mice were killed 30 min later Radioactivity recovered dependent line 1 O autonomous line 1 □ autonomous line 3 △ A) whole tumor B) tumor nucleus Each value shown is mean of two or more experimental results (From BRUCHOVSKY et al 1975a)

radioactivity into whole tumors (Fig 12A) increases as a linear function of dose of radioactive testosterone with little difference among the three lines However measurement of the rate of incorporation of radioactivity into nuclei (Fig 12B) uncovered striking differences in androgen transport across the nuclear membrane The rate of incorporation is maximal in dependent line 1 at 10×10^{-4} dpm/30 min/nucleus in autonomous line 1 at 14×10^{-4} dpm/30 min/nucleus but in autonomous line 3 at only 2×10^{-4} dpm/30 min/nucleus A comparative study on the nine variant lines listed in Table 5 again failed to disclose any meaningful differences in the incorporation of androgens into whole

tumor following a single injection of 200 µCi (4 6 nmoles) of radioactive testosterone (Table 4). On the other hand, transfer of androgens into nuclei is marked by a 47-fold difference between the highest rate (14.1 x 10^{-4} dpm/30 min/nucleus) and the lowest (0.3 x 10^{-4} dpm/30 min/nucleus). Both dependent lines demonstrate a high rate of transport but only two of the seven autonomous lines are characterized by this property. Furthermore, it may be seen that an apparent low rate of androgen transport is confined to the autonomous condition.

CYTOPLASMIC RECEPTORS AND INCORPORATION OF ANDROGENS

To establish whether as a general rule the transport of androgens across the nuclear membrane is dependent on androgen binding to cytoplasmic receptors, a comparative analysis of intracellular binding was carried out with all variants of the Shionogi mammary carcinoma. In these experiments, tumor bearing mice were injected intravenously with 200 µCi (4.6 nmoles) of radioactive testosterone and 30 min later the mice were killed. Cytosol fractions of tumors were then analysed for content of androgen receptors using gel-exclusion chromatography with Sephadex G-200 and sucrose-density gradient centrifugation. As exemplified by the results shown in Figure 13A, chromatography of cytosol protein from dependent lines 1 and 2 yields a large receptor peak in fractions 30-40 characterized by a sedimentation coefficient of 5.1 S in a linear 5-20% sucrose gradient containing 600 mM NaCl (Fig. 13B). The concentration of cytoplasmic receptor is estimated at 40 femtomoles/mg protein equivalent to about 1000 molecules per cell.

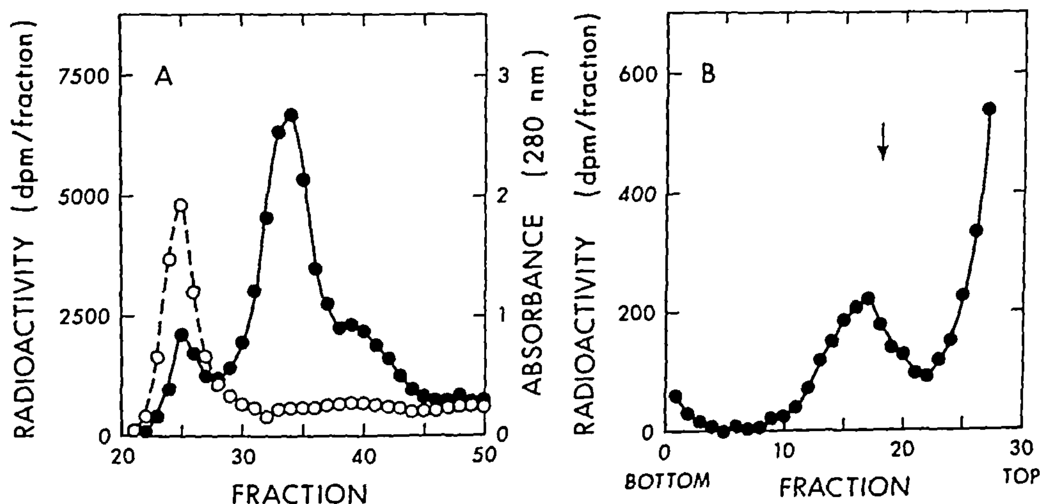


Fig 13 Cytoplasmic receptor and incorporation of androgens Male animal with a tumor of dependent line 1 was injected i v with 200 µCi (4.6 nmoles) of [1, 2-³H] testosterone In both cases cytosol was isolated according to method of BRUCHOVSKY et al. (1975c) and precipitated with ammonium sulfate at 80% saturation. Precipitate was analyzed for binding by Sephadex G-25 G-200 dual-column chromatography and by sucrose density-gradient centrifugation Column effluent was collected in fractions of 1 3 ml Absorbance at 280 nm, O Radioactivity recovered, ●. A) analysis by gel-exclusion chromatography B) analysis by sucrose density-gradient centrifugation Arrow marks position of albumin sedimentation standard

Autonomous lines 1 and 2 are also characterized by the presence of a cytoplasmic receptor as shown by the example in Figure 14A; but in the remaining autonomous lines no cytoplasmic receptor is detected (Fig 14B and Table 6) It follows from these observations that first

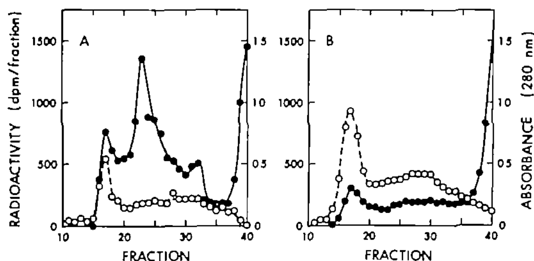


Fig 14 Cytoplasmic receptor in autonomous tumors Female mice with tumors of autonomous lines 1-7 were injected i v with 200 µCi (4 6 nmoles) of [1 2-³H] testosterone and killed 30 min later Protein precipitate recovered from cytosol was analysed by gel-exclusion chromatography with Sephadex G-200 (single column only) Absorbance at 280 nm O Radioactivity recovered ● A) autonomous line 2 with receptor B) autonomous line 4 without receptor

Table 6 Androgens and androgen receptors in Shionogi mouse mammary tumors

Tumor line	Radioactivity dpm/nucleus x 10^4	Cytoplasmic receptor
Responsive		
1	10 1	+
2	5 6	+
Unresponsive		
1	14 1	+
2	7 7	+
3	2 5	-
4	0 8	-
5	0 7	-
6	0 7	-
7	0 3	-

Male animals with responsive tumors and female animals with unresponsive tumors were injected i v with 200 µCi (4 6 nmoles) of [1 2-³H] testosterone and the mice were killed 30 min later The tumors were fractionated into cytosol and nuclear samples by centrifugation techniques (BRUCHOVSKY et al 1975c) and each sample was analyzed for content of radioactivity In addition cytosol protein was prepared as described in the legend to Table 4 and subjected to Sephadex G-25 G-200 dual-column chromatography for detection of cytoplasmic receptor

whether a tumor is dependent or autonomous cannot be decided accurately on the basis of the concentration of cytoplasmic receptor, and second, that the capacity to transport androgens across the nuclear membrane appears to be related to the presence of this molecule. Indeed, about 6000 androgen molecules/ 30 min/nucleus are transferred in cells possessing the receptor positive phenotype. As in prostate, there is a significant difference between the number of androgen molecules transferred into the nucleus and the number of detectable cytoplasmic receptors.

ANDROGEN-CHROMATIN INTERACTIONS AND NUCLEAR RECEPTOR

Once hormone is incorporated into the nucleus of a hormone-sensitive cell, theoretically, part of the hormone should be bound to a nuclear receptor But in analyzing extracts of nuclei from Shionogi mammary carcinoma cells labeled in vivo by the injection of radioactive testosterone, no peak of binding corresponding to the expected receptor is observed (Fig. 15). The first peak in Figure 15 represents binding of androgen to a large molecular weight component of the nucleus, presumed to be chromatin, and the second peak represents free steroid Analysis of extracts by sucrose-density gradient centriguation (Fig. 16) also fails to demonstrate any binding except to chromatin which is recovered at the bottom of the tube. Extensive efforts to release receptor from chromatin have been unsuccessful (BRUCHOVSKY et al., 1975c); thus it seems reasonable to conclude that in androgen-dependent lines of the Shionogi mammary carcinoma, androgens bind directly to chromatin with no receptor being involved in this interaction

The foregoing results strongly suggest that DNA synthesis and cell proliferation can be initiated in the absence of nuclear receptor

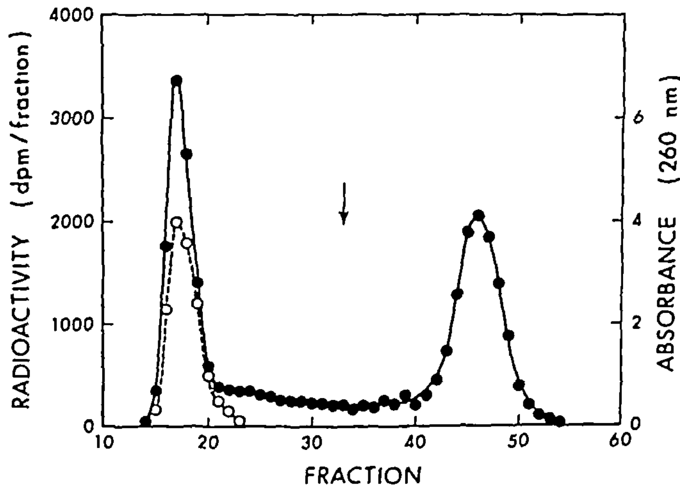


Fig. 15 Androgen-chromatin interactions and nuclear receptor Male and female mice with dependent and autonomous tumors respectively were injected i v with 200 μCi (4 6 nmoles) of |1, 2-³H| testosterone and killed 30 min later Nuclei from each tumor were extracted with 600 mM NaCl and extract was analyzed by gel-exclusion chromatography with Sephadex G-200 (single column only). Fractions of 1 3 ml were collected at a flow rate of 3 ml/h Absorbance at 260 nm, O. Radioactivity recovered, ● Arrow marks position of 24 Å, 3.3 S receptor characteristic of normal target cell nuclei Example shown is result with dependent line 1

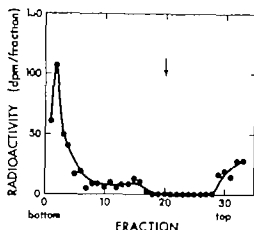


Fig 16 Analysis of nuclear binding by sucrose density-gradient centrifugation Nuclear extract was prepared as described in the legend to Figure 15 and treated with dextran-coated charcoal A sample of treated extract was then analyzed by sucrose density-gradient centrifugation Radioactivity recovered ● Arrow marks position of hemoglobin sedimentation standard

apparently by the direct binding of androgens to chromatin The lack of nuclear receptor is possibly not surprising for the following reason The Shionogi mammary carcinoma while dependent on androgens for growth does not actively regress in the majority of cases when androgens are withdrawn Consequently these findings also affirm the impression that nuclear receptor may be involved in the autophagic response

CORRELATIONS BETWEEN HORMONAL MECHANISMS AND RESPONSIVENESS

The failure of the estrogen receptor test to predict unequivocally whether a breast cancer will regress during the course of endocrine therapy has prompted us to survey the spectrum of potential hormonal responses and define these in very general terms Considering the primitive state of our understanding of hormonally-induced reactions present attempts to draw correlations between certain biochemical markers and specific phases of responsiveness unfortunately will be rewarded by superficial insights Nevertheless it seems worthwile to make a preliminary judgment on the significance of various correlations if simply to provide a broader basis for future discourse Thus we have classified correlations between potentially affiliated mechanisms into weak and strong categories as summarized in Table 7 Cytoplasmic receptor must be considered weakly correlated to nuclear receptor until the equivalence of the two molecules is confirmed Whether this proof can be easily obtained probably depends on the number of forms of cytoplasmic receptor in a particular tissue and on the changes which may occur in the configuration of cytoplasmic receptor during different stages of responsiveness On the basis of the results in Figure 10A it seems reasonable that nuclear receptor should be only weakly correlated to the initiation of DNA synthesis and to the negative feedback effect

Evidence has been presented (Tables 4 and 6) supporting the idea that the presence of steroid in the nucleus is stronlgy correlated to the presence of cytoplasmic receptor Therefore either the synthesis of

Table 7 Correlations between receptors and hormonal responses

Weak correlations	
[a] cytoplasmic receptor....	nuclear receptor
nuclear receptor........	DNA synthesis (initiation)
nuclear receptor.	DNA synthesis (negative feedback)
Strong correlations	
cytoplasmic receptor....	nuclear steroid
nuclear steroid.........	DNA synthesis (initiation)
nuclear receptor........	autophagy

[a] See text.

cytoplasmic receptor is induced by steroid, or the transport of steroid into the nucleus is expedited by cytoplasmic receptor. There is also a strong link between the presence of steroid in the nucleus and the initiation of DNA synthesis (Fig. 15). Finally, the disappearance of nuclear receptor and the onset of autophagia seem to be related catabolic events (Fig. 11).

The various strong and weak correlations summarized in Table 1 are significant when one attempts to predict responsiveness. In this context the chief points of relevance are shown in Figure 17 and may be interpreted as follows. The detection of cytoplasmic receptor in a cell suggests that steroid is being transported into the nucleus where it would be expected to stimulate new rounds of cell division Interruption of this pathway arrests tumor growth. The formation of a nuclear receptor would increase the probability that the cell will undergo an autophagic response if steroid is withdrawn. However, the state of the genome itself is an important factor, indeed if malignant transformation affects the initiator, nullifier, and autophage genes, responsiveness will be less predictable depending on the number of genes affected

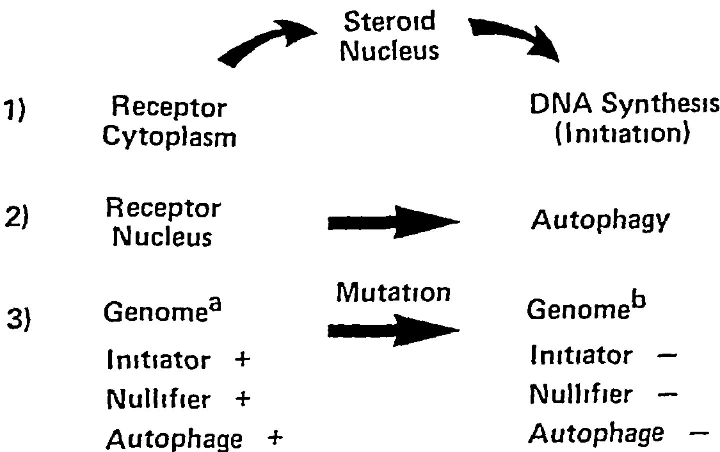


Fig 17. Significance of correlations

FUTURE RESEARCH ON HORMONAL RESPONSIVENESS

A major obstacle in predicting responsiveness accurately at the present time is the similarity in the receptor status of responsive tumors and of unresponsive type 2 tumors depicted in Figures 4B and 4D respectively More complete knowledge of the interaction of steroids and chromatin might provide a basis for distinguishing between such tumors

The unresponsive state may also be characterized by the inability of hormones to penetrate the cell nucleus as shown in Figure 4C Methods for overcoming the permeability barrier imposed by the nuclear membrane would seem to be desirable since the entry of steroid into the nucleus of an unresponsive type I cell might successfully result in activation of dormant regulatory genes and enable some measure of control over the growth of unresponsive cancers

In view of the importance of negative feedback in controlling cell proliferation (Fig 1) and in view of the very strong possibility that this mechanism is under direct hormonal control there appears to be an urgent need to evaluate the potentially undesirable consequences of prolonged therapy of cancers with antihormonal agents

SUMMARY

The failure of the estrogen receptor test to predict unequivocally whether a breast cancer will respond to endocrine therapy has prompted us to re-examine the spectrum of responses that might be expected in a hormone-sensitive tissue Three basic responses are recognized initiation of DNA synthesis and cell proliferation; negative feedback; and autophagia The expression of these responses may be partly or totally deficient in tumors In some tumors resistance to hormone may result from the lack of entry of hormone into the nucleus; in others the interaction of hormone with chromatin is probably abnormal

Evidence is presented in support of the idea that the presence of steroid in the nucleus is strongly correlated to the presence of cytoplasmic receptor The results also suggest that there is a strong link between the presence of steroid in the nucleus and the initiation of DNA synthesis Finally the disappearance of nuclear receptor and the onset of autophagia seem to be related catabolic events

ACKNOWLEDGMENTS

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Chapter 12
The Role of Prolactin in Breast Cancer

H G FRIESEN

Before addressing myself to the problem of the role of prolactin in breast cancer it may be helpful to review some of the physiologic variables which influence prolactin secretion on the one hand and prolactin receptors on the other Both parameters are important because hormone action depends on both serum hormone concentrations as well as on the number and status of tissue receptors for that hormone There are at least three hormones in man which are known to influence the breast placental lactogen pituitary growth hormone and prolactin All three hormones in humans exhibit prolactin-like effects and all must be considered when reviewing the role of prolactin in breast cancer

In the case of prolactin there is considerable heterogeneity in the circulating form of prolactin with at least three molecular weight forms of prolactin recognized (AUBERT et al 1975) A very large molecular weight form big big prolactin is seen rarely in patients who have pituitary tumors Secondly somewhere between 10 and 25% of serum prolactin in the circulation is big prolactin which is twice the apparent molecular weight of the monomeric form This is the major form of prolactin that exists both in serum and in pituitary extracts and is referred to as little prolactin During pregnancy there is a small increase in the proportion of big prolactin All three of these forms are recognized by conventional radioimmunoassays The forms of circulating prolactin can also be assessed using a radioreceptor assay for prolactin This assay recognizes the biologically active portion of the molecule Big big prolactin as judged by receptor assay is virtually inactive biologically whereas big prolactin has about 75% of the activity of the monomeric form which is taken to be 100% There has been at least one study which has suggested that in the resting state there is a larger proportion of the big prolactin than little prolactin in serum but others have not confirmed this finding

A number of physiologic variables which influence prolactin secretion are summarized in Table 1 It is important to recognize these particularly when epidemiologic studies are contemplated A circadian variation in prolactin secretion was reported by SASSIN et al (1973) who demonstrated that there is a significant increase in serum prolactin during sleep Prolactin is not secreted uniformly but appears to be released in bursts resulting in minor oscillations in serum levels of prolactin and the amplitude of the oscillations are enhanced even further during the night Again if epidemiologic studies are contemplated these facts must be taken into account in trying to optimize the sampling time and the interpretation of the data Finally it is important to recognize that stress of various kinds will increase serum prolactin concentrations as well

Table 1. Physiological variables influencing serum prolactin

1. Heterogeneity	"Big big" "Big" "Little"
2. Fluctuations	(a) Circadian (sleep-related) (d) Episodic
3. Age	
4 Sex differences	Male Female - Menstrual cycle(?) - Pregnancy - Lactation
5. Stress	

Basal serum prolactin concentrations vary throughout life (FRIESEN, 1973). In the foetus, concentrations reach maximal levels just before delivery when they are approximately 200-300 ng/ml. Subsequently in the newborn period they gradually decline in a matter of weeks to reach childhood levels of 5-10 ng/ml. In the prepubertal child, the concentrations of serum prolactin in males and females are very similar. At puberty in females, there is a modest increase in prolactin concentration to levels around 8-9 ng/ml, whereas in the male they are slightly lower - 5-7 ng/ml. During pregnancy, there is a large increase in serum prolactin values, ranging between 100 to 200 ng/ml. In women who are not breast-feeding, serum prolactin levels subside in the postpartum period to reach nonpregnant values in a matter of weeks after delivery whereas in women who breastfeed there is a prolongation of the elevated serum prolactin concentration. A number of studies have suggested that in old age there is actually a slight increase in serum prolactin in the female. Others have claimed just the opposite so that in order to interpret epidemiologic studies on serum prolactin concentration, one must take into account age dependent effects.

The hypothalamus controls pituitary prolactin secretion presumably via an inhibitory factor called prolactin inhibiting factor (PIF) which is liberated into the portal circulation. There is also a serotoninergic system which stimulates prolactin secretion. A number of drugs are known to influence prolactin secretion; some of these act at the hypothalamic level, others act directly on the prolactin cells in the pituitary (FRIESEN, 1973). Chlorpromazine and other psychotropic agents appear to exert their major effects by influencing the effective catecholamine levels in the hypothalamus leading to a diminution of PIF secretion and ultimately to an increase in serum prolactin concentration measured peripherally. L-dopa, after its transport across the blood brain barrier, is converted into dopamine. The latter has just the opposite effect and in a normal subject will lower serum prolactin. There is also some recent evidence to indicate that there are dopamine receptors on the pituitary prolactin cells themselves and so perhaps L-dopa and other drugs may act directly by inhibiting prolactin secretion at the pituitary level.

Two other agents which have been widely used in testing the hypothalamic pituitary prolactin axis are thyrotrophin releasing hormone (TRH) which by a direct action on the prolactin cell stimulates prolactin secretion

An ergot alkaloid Bromocryptin (CB-154) inhibits serum prolactin secretion extremely effectively

Some of the pharmacologic agents which stimulate prolactin secretion have been linked to the development of breast cancer but the precise mechanism leading to the alleged increase in incidence of breast cancer is by no means clear (Boston Collaborative Drug Surveillance Program 1974) L-dopa and CB-154 are agents which have been tested in patients with breast cancer There is some evidence to indicate that in occasional patients these agents are effective but other studies have not confirmed these claims (FRANTZ et al 1973; ENGELMAN et al 1975; HEUSON 1974)

One other consideration which it is important to recognize when examining the evidence which might link prolactin and the development of breast cancer is that hypophysectomy does not always lead to an absence of serum prolactin; the serum concentrations decline to unmeasurable levels but then in a period of days there is an increase in serum prolactin to the basal levels observed preoperatively One explanation for this might be that the surgery was incomplete However a second possibility is that there are probably prolactin cells along the upper part of the stalk of the pituitary or perhaps prolactin cells in the sphenoid sinus which after surgery, have been liberated from the dominant inhibitory hypothalamic control and then begin to secrete at maximal rates

I should now like to review some evidence on the mechanism of action of prolactin and to try and illustrate to you that an important consideration in this regard is the prolactin receptor Steroid hormones as Dr WITTLIFF and Dr BRUCHOVSKY discussed appear to enter the cells and exert their influence by an interaction at a nuclear level Protein hormones (prolactin being one example of this) appear to interact with specific binding sites on the plasma membrane of the target cells (Fig 1) Subsequent to this interaction a series of events are initiated one of which might be casein synthesis In the case of a number of hormones the interaction of the hormone with its binding site or receptor is closely followed by an activation of the adenyl cyclase system In the case of prolactin this does not appear to be the case but we really are very ignorant of the events that follow the interaction of prolactin with its receptor and this is obviously an area of great importance which must be examined extensively in the future

The binding sites (receptors) in the membrane fractions can be measured by standard techniques and they demonstrate specificity for

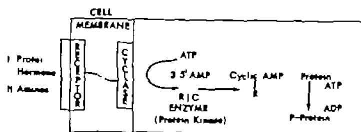


Fig 1 Schematic representation of mechanism of action of protein hormones and biogenic amines First interaction between hormone and target cell takes place when hormone binds with cell membrane In the case of many hormones this is followed by activation of adenyl cyclase However there is little evidence for this in the case of prolactin

prolactin or lactogens (SHIU et al., 1973). The displacement curves generated by prolactin-like hormones in an assay are very similar, presumably because the interaction of these hormones with the receptor is, in fact, very similar. Once again, human growth hormone, human placental lactogen, and human prolactin exhibit prolactin-like effects in the assay. On the other hand, sheep growth hormone and indeed all nonprimate growth hormones are similar to sheep growth hormone in that they lack prolactin-like effects. A variety of other hormones fail completely to interact with the prolactin receptor, thus the receptor is specific for prolactin.

The number of receptors or the number of binding sites in a target tissue can vary greatly, depending on the physiologic condition under which the tissue membranes are obtained (KELLY et al., 1976). For these studies we have used the rat liver as a model because we believe that some of the observations that are seen here are relevant to the mammary membrane receptor sites for prolactin as well (Fig. 2) In the foetus and neonatal period there are very few receptor sites in either male or female liver whereas with maturity, as the female approaches puberty, there is an increase to perhaps 50% of the maximal level seen in pregnancy in the female. In the male no difference is noted at puberty. Treatment of the male, on the other hand, with estrogens increases the number of prolactin receptors in rat liver membranes to reach levels found during the middle of pregnancy. Administration of estrogen either to the male or to the female has a similar effect. Pregnancy, as you can see, further increases the number of binding sites detected in the rat liver Hypophysectomy, on the other hand, has a very striking and opposite effect. Hypophysectomy of either the estrogen-treated male or hypophysectomy of the female within a short period of time, 12-24 h, results in a very major reduction in the number of prolactin receptors in the rat liver. Castration of the male has the same effect as estrogen administration to the male. POSNER et al. (1975) demonstrated that the implantation of pituitaries under the kidney capsule (RP) was able to induce prolactin receptors in rat liver membrane in male rats and indeed subsequently they and others have shown that the administration of prolactin can itself induce its own receptors (COSTLOW et al., 1975). In other words, there is an autoregulation of the

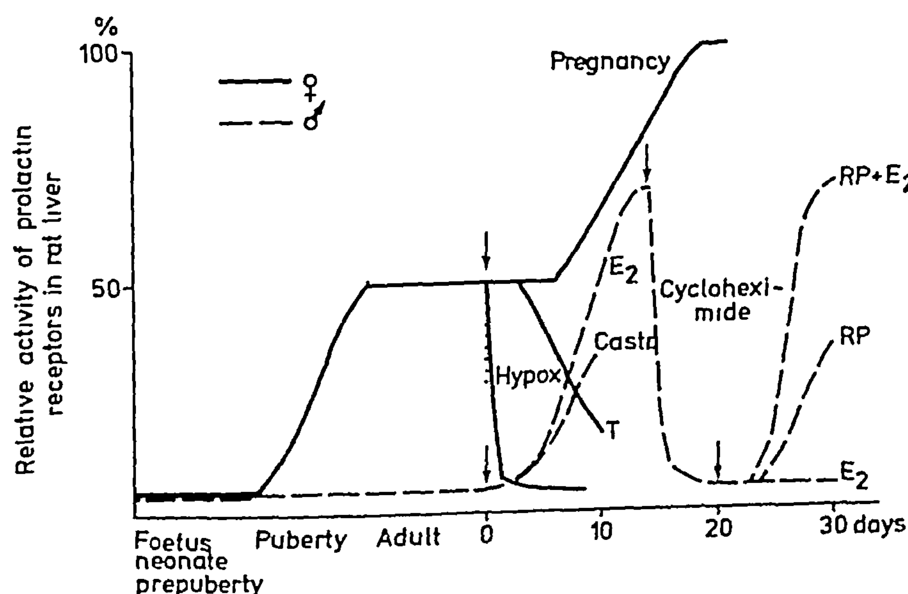


Fig 2. Variations in binding of prolactin to membrane fractions derived from rat liver under various experimental conditions

prolactin receptor by prolactin itself In this figure there is a lot of data summarized but the reader need not concern himself with the details except to carry away with him the notion that hormone receptors themselves vary and that presumably the biological effectiveness of the hormone depends at least in part on the number of prolactin receptors We indicated earlier that the hormone concentrations in the circulation vary and we have shown I think convincingly that the number of receptors in target tissues also can vary Hence we feel that in order to assess the biological effectiveness of a hormone one must know something about the serum concentration of the hormone and the number and status of receptors on target tissues

I should now wish to review the evidence linking prolactin and breast cancer In several experimental animal models there is fairly convincing indeed I think overwhelming evidence to indicate that prolactin plays a critical and major role in the development of mammary tumors In man the evidence is much less direct and much less convincing It is at best suggestive For example one can cite evidence like the fact that 30-40% of patients with breast cancer undergo remission for a period of time after hypophysectomy the implication being that it is the lowering of serum prolactin which is beneficial As I have already indicated hypophysectomy does not always lead to a striking and permanent reduction in serum prolactin; it may be only a very transient drop Serum prolactin has been shown to be elevated in a number of family members but not in patients with breast cancer This initial study by KWA et al in the Netherlands (1974) has very recently been confirmed in a similar study by HENDERSON et al in California (1975) in that they showed there is a significantly higher concentration of serum prolactin in the daughters of patients with breast cancer than in control subjects More direct evidence indicating that serum prolactin can have an effect on tumor slices was obtained by HOBBS et al (1973) who showed that when they incubated tumor slices from patients with *human breast cancer in the presence of prolactin, a significant* number responded using histochemical changes as an index of response That of course does not prove that in vivo prolactin is exerting a similar effect L-dopa and CB-154 as mentioned earlier have been utilized in an attempt to treat patients with breast cancer (FRANTZ et al 1973; ENGELSMAN et al 1975; HEUSON 1974) I think the evidence of a beneficial effect could be fairly summarized as being controversial perhaps even less than convincing although there have been occasional cases in which objective regression has been claimed In most of these reports the effect on bone pain has been much more striking than the effect on objective tumor regression

In animal models FURTH (1972) has shown that prolactin can act as a cocarcinogen in the development of experimental mammary tumors in mice and rats Furthermore PEARSON showed a number of years ago that the dimethylbenzathracene (DMBA)-induced rat mammary tumor was highly dependent on prolactin (PEARSON et al 1969) KELLY et al (1974) examined whether there was any correlation between the number of prolactin receptors and the responsiveness of the tumor to endocrine manipulation Tumors were induced by DMBA followed by the administration of prolactin to identify hormone-responsive tumors These tumors subsequently were removed and prolactin receptors were determined in individual tumors There was a significant correlation between the specific binding or the number of receptors for prolactin and the growth index that is the increase in size of the tumor following the administration of prolactin The tumors with the greatest growth index had the highest number of prolactin receptors

We then wondered whether a similar approach might be utilized to identify prolactin-responsive human mammary tumors As mentioned earlier in

this conference, the measurement of estrogen receptors has been very helpful in identifying endocrine-responsive tumors. In our study we examined a series of 48 human mammary tumors and 12 benign lesions. (HOLDAWAY et al., 1975). Subsequently an additional 20 tumors have been examined which show results that are very similar. The tumors were homogenized and membranes were obtained or slices were made to examine the binding of prolactin, insulin, and growth hormone along with the binding of estrogen in the cytosol of these tumors. Because the availability of tumor tissue is often limited, we utilized a micro-slice technique in which cryostat-cut sections are incubated in vitro in the presence of labeled hormone (HOLDAWAY and FRIESEN, 1976). With this technique, one can use as little as 10-20 mg of tissue which is a considerable improvement on the membrane technique.

Approximately 20% of the subjects studied exhibited significant binding of prolactin if one takes a cut-off of about 1% as being perhaps equal to the blank value. None of the nonmalignant mammary tissues bound either prolactin or growth hormone (Table 2).

The binding sites appeared to be class specific in that human, bovine, and ovine prolactin as well as human placental lactogen and human growth hormone inhibit the binding of the human prolactin tracer. Other hormones failed to displace the binding of the tracer. It is of interest that HOBBS and his colleagues, using their in vitro incubation technique, have very recently reported on a patient with breast cancer who exhibited hPL responsiveness. The patient had a mammary tumor which grew extremely rapidly during pregnancy which then regressed promptly when the pregnancy was interrupted and HOBBS was able to show that the tumor was highly responsive to human placental lactogen in vitro (BARRETT et al., 1975).

In our series, in addition to binding of lactogens, very high insulin binding was detected but in this case both malignant and nonmalignant breast tissue bound insulin equally.

Table 2 summarizes the distribution of hormone binding sites in this study. You can see that approximately 60% of malignant tumors had binding sites for estrogen. Of these tumors, 20% also bound prolactin, 85% bound insulin, and only 4% had growth hormone binding sites. It is also apparent that some tumors had binding sites for all four hormones. Perhaps it is these particular tumors that are most responsive to endocrine manipulation.

In conclusion, the data attempting to link human breast cancer with prolactin remains indirect and the overall evidence is less than compelling. Nevertheless, in the face of unanswered questions and uncertainty it is clear that additional research is required. Since so much of the "hard" evidence ultimately rests on attempts to demonstrate changes in the clinical course of the disease with changes in serum prolactin levels, it is imperative that cognizance be taken of the

Table 2. Incidence of hormone receptors in malignant human mammary tumors

	%Positive
1. Estrogen	60
2. Insulin	80
3. Prolactin	20
4. Growth hormone	4

variables which influence prolactin secretion Moreover since hormone action depends on serum levels as well as on tissue responsiveness a better means of assessment of the latter must be sought It may be that one index of the latter is provided by determing the number and status of the receptors for the hormone in this case prolactin It is still too early to say whether this will be a helpful approach

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Chapter 13
Some Thoughts Concerning the Primary Therapy of Breast Cancer*

B FISHER

When consideration is given to recent advances and progress made in the treatment of cancer, little attention is directed toward those changes which are taking place that are probably as profound and as of far-reaching in consequence as are any in our time. To many - particularly those in other disciplines - the surgical approach to cancer is deemed anachronistic in concept. While categorically such may be true, the leading edge of that speciality is attempting to redefine the basis for cancer surgery so that it is in keeping with present understanding of tumor biology.

Just as repair of a hernia is based upon anatomical considerations and operations for duodenal ulcer upon physiologic principles, so must there be a firm rationale for the surgical management of cancer. For almost a century those precepts upon which cancer surgery were and still are based have been almost entirely without opposition - perhaps for want of viable alternatives, or perhaps because of the dominance of those in positions of leadership. Within the last decade partially because of dissatisfaction with results obtained, but more importantly because of new information coming from a variety of sources, old "unchallengable" concepts have been reassessed. In many instances they have been found lacking. Consequently, there has resulted the present period of clinical uncertainty which will persist until a firm new basis for cancer surgery has evolved and until its new position in the "family" of modalities available for the treatment of cancer has been established. Surgery no longer enjoys the distinction of being "an only child" in that regard.

The principles upon which present day cancer surgery is based were formulated almost 100 years ago (HALSTEAD, 1890). No one was more influential in conditioning the minds of generations of surgeons relative to the management of patients with neoplasms than was the American surgeon, WILLIAM S. HALSTED. While his name is most closely associated with the operation for breast cancer popularly referred to as "radical mastectomy" the same precepts promulgated by him for that procedure have served as an enduring basis for all cancer surgery. In order to understand Halsted's rationale for the type of surgery he advocated, it is important to appreciate his concept of the biology of cancer - and particularly how he thought tumors disseminated. Some insight relative to that is gleaned from one of his publications (HALSTED, 1907). He apparently placed little significance on the bloodstream as a mechanism for the development of metastases.

Two other precepts harmonized with the "Halstedian" concept in giving rise to "modern" cancer surgery. First, it was (and still is by many)

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supposed that a growing tumor remains localized at its site of origin for a period of time but at some instant during its growth tumor cell invasion of lymphatics and dissemination of regional nodes take place After a further interval during which time the tumor is loco-regional only and during which time there is an increase in tumor size systemic dissemination ensues Second as a result of the theory first formulated by VIRCHOW (1860) there has existed the belief that lymph nodes provide an effective barrier to the passage of tumor cells

As a consequence of the above considerations and in keeping with the understanding of the disease at the time there arose an anatomical basis for cancer surgery The proper cancer operation consisted of removal of the primary tumor together with regional lymphatics and lymph nodes by en bloc dissection Since it was deemed that there was a certain orderliness about tumor spread and that clinically recognizable cancer was in many instances a loco-regional disease it was considered to be more curable if the surgeon would only be more expansive in his interpretation of what constituted the region and if above all he utilized better technic so that he could eradicate the last cancer cell Loco-regional recurrences were more often than not considered to be the result of inadequate application of surgical skill rather than a manifestation of systemic disease The hope was held high that one more lymph node dissection would cure more cancers Radical cancer surgery based upon those anatomical considerations has persisted for 75 years

The past quarter of a century has seen the zenith and the beginning decline of cancer surgery based upon anatomical principles Despite, in some instances extraordinary feats of skill and daring noteworthy gains in terms of patient survival and disease-free life have eluded the surgeon To the contrary much physical and mental trauma has been inflicted without those dividends Partially as a result of disappointment with results obtained and more significantly as a consequence of conceptual changes that have resulted from new information concerning tumor biology a new basis for cancer surgery is undergoing synthesis What considerations are leading to a redefinition of the role of operation in the treatment of cancer? The following are of significance in that regard

A BREAST CANCER AS A SYSTEMIC DISEASE

Information from a variety of sources indicates that most if not all patients with solid tumors have disseminated disease by the time a clinical diagnosis is established This is not surprising when it is appreciated that a 1 cm tumor which is usually the minimal size capable of physical diagnosis (e g breast) and which is looked upon as an early tumor has already progressed through 30 of its 40 doublings that number which is lethal to the patient Considering that relatively few tumors are detected when they are less than 1 cm it is appropriate that they may be considered advanced at the time of diagnosis Data recently reported (FISHER et al 1975b) regarding the percent of treatment failures 10 years following radical mastectomy for what were considered to be clinically curable breast cancers strikingly emphasizes the systemic nature of that cancer (Table 1) The finding that 3 of 4 patients with positive axillary nodal involvement and that almost 9 of 10 with 4 or more of such nodes containing tumor become treatment failures indicates the inadequacy of extensive loco-regional surgery Treatment failure rates of other common and lethal cancer

Table 1 Treatment failures 5 and 10 years post-radical mastectomy

Nodal status[a]	5 Years (%)	10 Years (%)
Positive and negative (all patients)	40	50
Negative	18	24
Positive	65	76
1-3	50	65
> 4	79	86

[a] Histologic

i.e., pancreas (99%), lung (90%), prostate (70%), and bowel (60%) similarly indicate the probability of metastases at the time of diagnosis

Correlating information concerning the fate of patients having breast cancer with that which is known about growth rates and other features regarding the kinetics of cells from such tumors, and employing certain assumptions, SKIPPER (1974) has provided estimates of the residual tumor cell burden that might be expected to be present in a host following primary tumor removal, i.e , the number of viable cancer cells that are beyond the reach of surgery (Table 2) Zero viable tumor cells in that chart refers to none above some relatively small number with which host immune mechanisms may be able to cope. He has also provided some "reasonable" estimates of the body burden of tumor cells present at detection of a recurrence (Table 3). As a result of such "modeling" there arises some insight into the prevalence of cancer as a systemic disease at diagnosis and the numbers of cells which need to be eradicated by systemic therapy to enhance the curability of surgery.

The fact that some patients are apparently "cured" by operation alone is no indication that the surgical procedure eradicated every last cancer cell, that the disease was completely loco-regional in extent, and that dissemination had not taken place. Such a concept is most difficult for the surgeon to accept. There is failure to appreciate that the residual tumor cell burden may have been sufficiently minimal for its eradication by host factors which play a significant role in the success or failure of the operative procedure. It is impossible to estimate the number of micrometastases which may have been aborted by removal of a primary tumor. That removal of a primary tumor is not equivalent to the removal of a "foreign body" is apparent by the increasing number of reports describing a variety of changes in the host and in residual tumor cells. The following sections consider those alterations.

B. CONSEQUENCES OF REMOVAL OF A PRIMARY TUMOR

Not only are host immunologic mechanisms affected by a growing tumor, but its removal may so further alter those host functions as to influence the course of a patient. The consequences of both the tumor removal and the surgical procedure itself seem important in that regard. In addition, there is evidence to indicate that removal of a primary tumor may alter the growth pattern of micrometastases.

Table 2 Residual tumor cell burden following primary tumor burden

Viable breast cancer cells beyond the reach of surgery	Percent of operable patients bearing numbers of tumor cells indicated presuming an overall median doubling time of 30 or 40 days (and other stated assumptions)					
	Negative nodes		1 or More positive nodes		4+ Positive nodes	
	30-day	40-day	30-day	40-day	30-day	40-day
0 or 1 slow cell	65[a]	65[a]	36[a]	36[a]	14[a]	14[a]
1 (with median DT) or <	80	74	56	47	22	17
10^1 or <	82	77	59	51	26	18
10^2 or	85	80	63	56	30	22
10^3 or	88	82	69	59	39	26
10^4 or <	90	86	73	65	45	33
10^5 or <	93	89	78	70	52	42
10^6 or <	95	93	83	78	63	53
10^7 or	97	96	89	85	76	67
10^8 or <	99	99	94	92	88	85
10^9 or <	100	100	100	100	100	100

[a] 5- Year tumor-free survivors observed From SKIPPER (1974)

Table 3 Seemingly reasonable estimates of the body burden of tumor cells at detection of recurrence

No of tumor cells	Weight	Diameter (mm)	Palpable (depending on site)
10^1	0 01 µg	0 025	No
10^2	0 1 µg	0.06	No
10^3	1 0 µg	0.12	No
10^4	10 µg	0 25	No
10^5	100 µg	0 6	No
10^6	1 mg	1.2	No
10^7	10 mg	2.5	No
10^8	100 mg	6	?
10^9	1 g	12	Yes
10^{10}	10 g	25	Yes
10^{11}	100 g	60	Yes
10^{12}	1 kg	120	Yes

The volume of a sphere = $0.5236d^3$

Subcutaneous solid animal tumors are usually first palpable and measurable at about 60 mg

[a] Reasonable range. the average is likely to be somewhere between 1 and 10 g.

From SKIPPER (1974)

1 Immunologic Effects

In general it is accepted that tumors are antigenic and are capable of eliciting a host immune response which is both cell and serum mediated Peripheral blood lymphocytes from patients with a variety of tumors have been found to be capable of killing tumor cells of the same tumor type growing in tissue culture Removal of a primary tumor has a diversity of effects on the various aspects of the host immune response Serum from patients with a variety of tumor types has been shown to be capable of interfering with the cytotoxic effects of lymphocytes Whatever the mechanism(s) responsible i e blocking antibody hindering lymphocyte/tumor cell interaction by coating the latter antigen-antibody complexes in the serum interfering with cell mediated responses or antigen released from tumor cells interfering with lymphocyte action by binding to their surface evidence indicates that the surgical removal of a tumor diminishes the inhibitory activity of serum directed toward cellular immunity Removal of a primary tumor has also been observed to affect cells involved in the immune process in a variety of ways Moreover circulating antitumor antibodies specific for a particular immunizing tumor were detected in an immune host by PILCH and RIGGINS (1966) only after surgical removal of a tumor

2 Effect of Removal of Primary on Residual (Metastatic) Tumor Cell Kinetics

Numerous studies (FRINDEL et al 1967; LAIRD 1964; McCREDIE et al 1965; DEWYS, 1972) have demonstrated that as tumors increase in mass there is a slowing of their growth rate This growth pattern of solid tumors has been described mathematically by means of the Gompertzian equation which expresses the rate of tumor growth and the effect of a retarding factor that fits the decreasing rate of growth as the tumor increases in size Thus solid tumor growth has been characterized as gompertzian Of prime importance is how the presence or removal of a primary tumor influences the kinetics of metastatic tumor cells for such information has direct therapeutic implication According to SCHABEL (1969) when tumors increase in size the growth fraction of the viable tumor cells in cell division cycle decreases Large tumors are apt to contain a preponderance of resting tumor cells i e those with a prolonged G_1 time DEWYS (1972) has reported a synchronous slowing of the rate of growth of an implanted tumor as well as of its metastases even though the latter were microscopic in size With removal of the primary tumor the slowing of metastatic tumor growth was reversed Thus a significant reduction of a large viable tumor cell population by operation or radiation or subcurative chemotherapy probably results in an increase in the growth fraction and shortening of the tumor cell generation time in the metastases Temporarily nondividing or noncycling cells comprising metastatic foci may once again become actively cycling and dividing and thus become more vulnerable to chemotherapeutic agents (SCHABEL 1975)

C Reassessment of the Role of the Lymphatics and Lymph Nodes in Cancer

There has been renewed concern with the role of lymph lymph nodes and the lymph vascular system in the biology of malignancy (FISHER and FISHER 1968) Contrary to Sampson HANDLEY s and HALSTED s beliefs it is now well appreciated that tumor cells on gaining access to lymphatics may be carried as emboli directly to a lymph node where they are arrested in the subcapsular sinus of one or more lobules at which point early growth occurs (Fig 1) Likewise it is accepted that additional cells from a primary tumor may traverse collateral or alternative

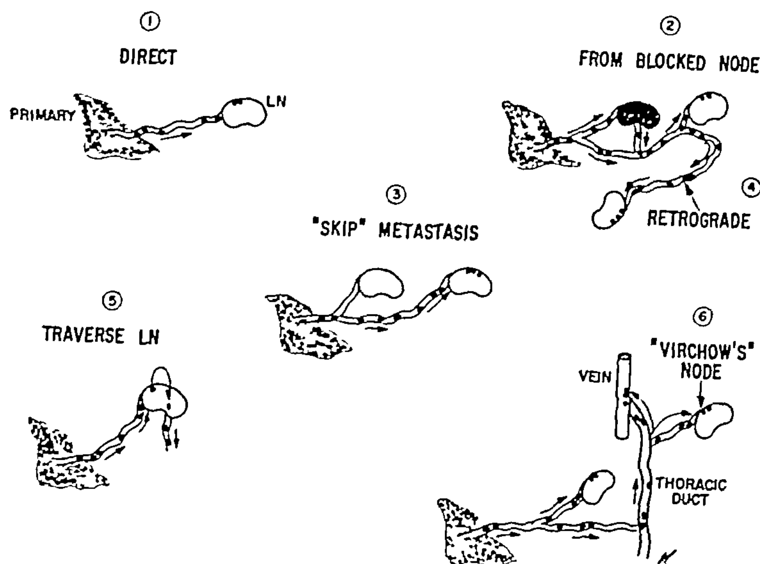


Fig. 1. Pathways of lymphatic tumor cell dissemination

lymphatic pathways to lodge in more distal nodes when those more proximally are already involved. That lymphatic metastases may appear in distant rather than proximal nodes, even when the latter are not involved, has been recognized since Paget's time in 1860 (PATEY, 1967) but is consistently ignored in surgical thinking. This phenomenon of skip metastases is related to direct lymphatic communication and the dynamics of the lymph flow in the area involved. Such bypasses can explain the noninvolvement of individual lymph nodes and atypical distribution of lymphogenous metastases.

The concept originally expressed by BARTELS (1909) that lymph does not reach the blood without passing through at least one lymph node still prevails, despite evidence to the contrary. It has been established that, in addition to being carried to regional lymph nodes, tumor cell emboli may bypass such nodes to enter directly into the thoracic duct and be conveyed directly to veins at the base of the neck, from which point they are blood-borne. Controversy has existed concerning the magnitude and significance of other lymphatico-venous pathways throughout the body. This has been particularly so relative to the presence of such connections in lymph nodes and the part they play in the entry of tumor cells into the bloodstream. Studies from our laboratory support the existence and possible importance of such communications in tumor cell dissemination. Evidence by others seems to substantiate such an event.

Recently, we have challenged the concept that lymph nodes act as an effective barrier to tumor cell dissemination. VIRCHOW (1863) first formulated the theory that the lymph node effectively trapped particulate matter in the lymph. Over the years, the effectiveness of nodes in trapping inanimate particles, bacteria, viruses, and red blood cells has been evaluated repeatedly. As a result of opinion and inference from such investigations, there exists, with few exceptions, the generally held belief that lymph nodes provide an effective barrier to the passage of tumor cells. It is remarkable, however, that this conclusion was reached without ever employing tumor cells to test the integrity of these lymph node relative to the transmigration of such cells. Data obtained by us in that regard support the conclusion that the lymph note is not as effective a barrier to tumor cells as formerly believed FISHER and FISHER, 1966a, 1967a; 1967b). The majority of tumor

cells entering the node may fail to maintain permanent residence Moreover information obtained with erythrocytes or other particulate matter has little relevance to the fate of tumor cells In addition there is evidence to suggest that tumor cells themselves could be as much a determinant of their residence as are the biologic and mechanical properties of the node

As a result of the foregoing it no longer seems tenable to believe that tumor cells in lymphatics have only one final destination - lymph nodes There is sufficient evidence that tumor cells that are primarily lymph-borne may reach the blood vascular system through which they become further dispersed Only recently however has it been demonstrated that tumor cells circulating in the blood vascular system may likewise find their way into the lymphatics and hence the thoracic duct (FISHER and FISHER 1966b) Thus the two vascular systems may be so unified insofar as tumor cell dissemination is concerned that it is no longer realistic to consider them independently as routes of neoplastic dissemination

Evidence implicating immunologic mechanisms in the fate of tumors provokes other considerations Should a human neoplasm contain tumor antigens that evoke a host immune response - a situation no longer to be considered remote - it would seem reasonable to anticipate that when cells from such a tumor become disseminated via lymphatics they may be destroyed by the immune node Experimental evidence obtained both in vivo and in vitro in support of the tumor cell destructive properties of sensitized lymphocytes supports such a possibility

As a consequence of all of these findings and considerations it is unrealistic to continue to accept the finding of negative lymph nodes as indicative only of the fact that a given tumor has been removed prior to its lymphatic dissemination

Similar considerations may be given to positive regional lymph nodes (Fig 2) Conventionally the presence of a tumor-containing node is considered simply to be the result of tumor cells having disseminated via lymphatics with their subsequent lodgment and growth in nodes This may represent an oversimplification of the phenomenon Perhaps only when the number of disseminated cells i e the challenge dose exceeds the capability of node for cell destruction or alternatively there is a reduction of the immune capabilities of the node and/or possibly a change in the biologic nature of the tumor cell does tumor grow in the lymph node

Recently we carried out a series of investigations that utilized lymph nodes from patients with operable breast and colon cancers Findings (FISHER et al 1972) support the possibility that the reason why some nodes in a group harbor metastatic tumor and others do not is that such metastases are more likely to be related to biological differences between nodes than to anatomic happenstance (i e transport of tumor cells to some nodes but not to others) as is conventionally believed

Thus the presence or absence of tumor in lymph nodes may not be of as great significance as was believed in the determination of lymphatic tumor cell dissemination Since as is well-known there is a better prognosis in cancer patients when lymph nodes are not involved on cursory consideration such a hypothesis may seem untenable It may be however that positive lymph nodes merely denote that disseminated tumor cells that produced such growth as a result of intrinsic cell properties and/or host factors are capable of developing metastases in other parts of the body as well Negative nodes may merely reflect conditions that in addition to preventing nodal growth of tumor also

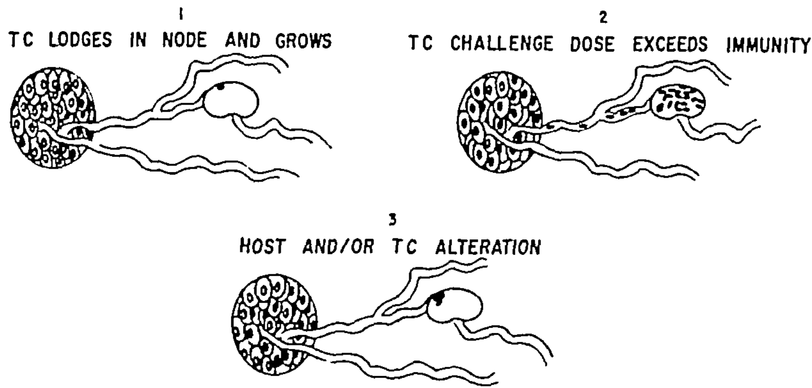


Fig 2. Alternative reasons for tumor-positive lymph node

inhibit metastases from occurring in other places. Moreover, perhaps those cells that traverse lymph nodes are more readily destroyed when they are dispersed via the blood vascular system than when they are retained in significant numbers in nodes. Might it not be possible that the patient with negative lymph nodes is the one whose immune competence is entirely adequate to eliminate disseminated tumor cells, thereby preventing secondary tumor growth and that this is the reason for a more favorable prognosis?

A BIOLOGICAL BASIS FOR CANCER SURGERY

As a result of the foregoing data and comments it would seem that if patient curability was dependent upon the surgical removal of "every last cancer cell," the outlook would indeed be bleak and operative intervention would at best be looked upon as a palliative procedure The primary aim of oncologic surgery at present seems to this reviewer to be directed toward reducing the tumor burden of the patient to a number of viable cells which are entirely destroyable (1) by host immunologic (and other) factors alone, (2) by systemically administered anticancer agents, or (3) by a combination of both. The increasing evidence indicating that primary tumor removal may result in a variety of beneficial host changes and may, by increasing the growth fraction of residual tumor cells, make them more susceptible to anticancer agents is of profound importance and provides a rational basis for cancer surgery.

BREAST CANCER SURGERY IN CONJUNCTION WITH OTHER THERAPEUTIC MODALITIES

There are two aims in the management of patients with breast cancer. In order of importance the first is related to achieving a disease-free life, and the second is directed toward attaining the best cosmesis and quality of life possible without compromising the patient's chance for cure. Until the present, arguments relative to the merits of less radical operative procedures have been directed toward testing the null hypothesis, i.e., that such procedures are as good but no better than more extensive operations relative to survival. Operations, for example, such as segmental or total mastectomy for breast cancer have been advocated essentially for improvement of cosmesis rather than

the prolongation of disease-free survival It has been hoped that the improvement in cosmesis could be achieved with a disease-free interval equal to that obtained by a more radical procedure i e as good or as bad depending upon one s satisfaction or dissatisfaction with results obtained by the radical operation Despite all that has been written and said despite all the emotional rancor that has been generated, it really has not been shown in a properly carried-out comparison that radical mastectomy is or is not better than any other procedure in comparable patients After 10 years of planning the NSABP began in 1971 such a clinical trial In that trial clinically negative node patients are randomized into three groups: radical mastectomy, total mastectomy followed by regional radiation and total mastectomy alone Those patients subsequently developing evidence of axillary node involvement have an axillary dissection Clinically positive node patients are randomized between radical or total mastectomy with radiation Before long definitive information regarding the results of that trial should be available

THE USE OF SYSTEMIC THERAPY

Since most if not all patients with breast cancer have disseminated disease at the time of diagnosis there is ample justification for the use of systemic therapy as a part of primary treatment Twenty years ago representatives of 23 institutions adopted a common protocol to determine the efficacy of administering chemotherapy in conjunction with curative cancer surgery to decrease recurrence and extend survival of patients with cancer of the breast This marked the beginning of the NSABP Women were randomized in double-blind fashion between two treatment groups: (1) conventional Halsted radical mastectomy and TSPA and (2) radical mastectomy with or without placebo (control) Patients received TSPA at the time of operation and on each of the first 2 postoperative days Direct recurrence and survival rate calculations were obtained from 826 acceptable study patients grouped according to nodal and menopausal status and results have been reported id detail (FISHER and FISHER 1968) Positive node patients were further subdivided into those having one to three or four or more such nodes At the end of 5 years there was no significant difference in recurrence rate between patients receiving TSPA or placebo in any of these six principal categories If however recurrence rates for TSPA and placebo groups were plotted against time following surgery significant information was revealed which was not obtained by examination of data only at the end of 5 years Whereas there was no significant difference between the two treatment groups at any time in five of the six menopausal and nodal categories a difference was discernible between the TSPA and placebo groups of premenopausal patients who had four or more positive nodes TSPA-treated patients in that category demonstrated a recurrence rate between the 18th and 36th postoperative months which was approximately 40% less than in the placebo group The effect of TSPA in this category was best demonstrated by the observation that 50% of patients in the placebo series had recurrences by the 13th month after surgery whereas recurrences did not occur in half of the TSPA patients until the 45th month of follow-up While the difference between the treated and control groups relative to treatment failure was no longer statistically significant after 5 years it was 20% A 33% difference in the survival rate that was significant ($p<0\ 05$) was observed at that time between the TSPA-treated and control groups: 56 5% for the former vs 24 3% for the latter Recently results obtained from a 10-year follow-up of patients entered into the TSPA study were reported (FISHER et al 1975b) The 10-year findings revealed a persistence of the difference in treatment failure and survival

rates. After 10 years, 21% fewer patients in the TSPA group had treatment failures and 21% more of them survived. Thus, it would seem that the initial suppression of treatment failures had a lasting effect that was reflected in patient survival. The data also suggested that the limited chemotherapy employed was more effective in patients having smaller tumors. Such a finding is in keeping with experimental observations and with modern concepts of chemotherapy which suggest that a therapeutic regimen should be more effective in a host with a minimal tumor burden.

In 1971, a clinical trial of the effectiveness of prolonged adjuvant chemotherapy was begun. Patients were randomized so that they either received placebo or L-phenylalanine mustard (L-PAM) for 5 consecutive days every 6 weeks.

Since a specific aim of that study was to ascertain whether the administration of L-PAM could prolong the disease-free interval of patients, when evidence to indicate that achievement had occurred (September, 1974) a progress report of findings were presented (FISHER et al., 1975b). At that time it was observed that treatment failures had occurred in 22% of 108 patients receiving placebo and 9.7% of 103 women given L-PAM ($P = 0.01$). A statistically significant difference ($P = 0.02$) existed in favor of L-PAM relative to disease-free interval

In premenopausal women, the difference with respect to disease-free interval of treated and control groups was highly significant ($P = 0.008$). A treatment failure occurred in 30% of premenopausal patients receiving placebo and 3% of those treated with L-PAM ($P = 0.008$). Whereas a similar trend was observed in postmenopausal patients, the difference was not statistically significant.

Of interest was the finding that results were achieved with minimal alteration of the well-being of patients despite the fact that 60% of women demonstrated myelosuppression (grades 1 and 2 toxicity). At that time it was pointed out that many questions were raised by the findings which only time could resolve. Would the differences in disease-free rates be sustained or would they only be transient? Would such differences be manifested in survival rates? Might undesirable sequelae of L-PAM therapy such as lymphoproliferative neoplasms only become apparent after prolonged follow-up study? Those questions and others are at present just as significant and remain to be answered

COMMENT

What is the significance of these findings? How do they affect current primary breast cancer management and how will they influence future therapeutic strategies against this disease? The most important aspect of the findings is not the statistical magnitude of the differences achieved with L-PAM, nor even that the results could be obtained with minimal toxicity. Their greatest importance is that for the first time it has been demonstrated from a well-controlled randomized clinical trial that the rationale for using prolonged chemotherapy as an adjunct to operation is a sound one. Different agents, muliple agents, as exemplified by the recent report of Bonadonna using CMF, refinements of administration, sequencing of drugs, etc., will undoubtedly produce increasingly better results. This will follow as a corollary to these observations, but the initial step forward has been achieved. The concept of the effectiveness of adjuvant chemotherapy has been proven. In retrospect these observations are supportive of the results of the 1958 study, which provided the first evidence that such adjuvant therapy might be meritorious.

With demonstration of the worth of systemic adjuvant chemotherapy all treatment categories directed toward local and regional tumor control have been elevated to a new level of accomplishment insofar as achieving a disease-free state The better results with L-PAM (or other chemotherapeutic regimes) following radical mastectomy are related to its effect on systemic disease That same systemic effect should be observed if L-PAM is given following total or segmental mastectomy Moreover the possibility exists that such systemic therapy may be equally effective against minimal residual local and/or regional disease As a consequence all modalities which have been considered for local and regional control must be evaluated and re-evaluated in the light of findings indicating the effectiveness of a systemic agent If for example even should total mastectomy not be as effective as radical mastectomy the possibility remains that with effective systemic therapy the procedures could produce equivalent results Similarly even if segmental mastectomy is at this time not as efficacious as total or radical mastectomy the addition of chemotherapy could make it an equivalent procedure In essence as systemic therapy becomes more effective the more likely it becomes that lesser operative procedures could be comparable As a consequence increased disease control may be accomplished together with better cosmesis It is not unreasonable to anticipate that the use of systemic therapy will make more remote the chance that a lesser surgical procedure will be putting the patient at a disadvantage In our opinion as a result of the L-PAM findings and others using systemic therapy evaluation of segmental mastectomy is more appropriate than before in those patients in which it is considered proper for trial Shortly the NSABP will conduct such a trial and invites participation

Finally it is this reviewer s opinion that the next decade may well represent the most critical and significant period in the history of breast (as well as other) cancer therapy The present spectrum of combined modality trials added to those which are proliferating at an ever more rapid pace throughout the world are setting the stage for that crisis situation Should many or most of those trials started with the most noble of intentions fail to be continued to a point where meaningful data are obtained or even worse because of their improper design or implementation produce information of questionable credibility valuable time will have been lost patient resources will have been squandered and above all therapeutic confusion and disenchantment will prevail On the other hand should a sufficient number of the protocols be impeccably carried to completion and meaningful data obtained the next 10 years could be even more crucial For there will result verification or repudiation of not only the concepts and principles upon which the use of adjuvant therapy is based but of the very worth of those modalities which at present represent our total therapeutic resources and hopes for cure of the diseases

If for example as unlikely as it may seem despite the multiplicity of trials evaluating different chemotherapeutic combinations in a variety of ways should a significant prolongation of freedom from disease which is reflected in a major improvement in survival fail to be observed the entire chemotherapeutic approach to the treatment of breast cancer will be subject to challenge The present regression of metastases for 6-18 months following combination chemotherapy in up to 85% of patients should not obscure the dismal fact that long term control for 3 or more years after the onset of chemotherapy is achieved in less than 15% of those treated for recurrence The 5-year survivorship after the onset of chemotherapy for recurrent metastases is less than 5% despite best efforts Consequently if adjuvant chemotherapy fails to make a dramatic impact on the disease free survival of patients

with "minimal" disease the outlook is indeed bleak for those with advanced tumors, unless, of course, new agents become available.

Similar considerations relate to the use of immunotherapeutic and hormonal agents. Obviously, unless they can aid in the "cure" of more "curable" lesions, their worth in advanced disease will be nil

More optimistically, it is likely that from the muliplicity of trials there will come information regarding which regimens are most effective against a particular subset of breast cancer patients with a minimum of toxicity. It is not unreasonable to predict that the same magnitude of chemotherapy employed in patients with ≥ 4 positive nodes having a highly unfavorable prognosis will not be required for the patient with one node positive. Most urgently needed for the proper synchronization of available therapeutic modalities so as to make such a concept a reality is the availability of a biological assay which can indicate with precision the amount and the location of residual tumor present following operation. It may be predicted that as information becomes available from more of the trials currently in progress the physician caring for such patients will be faced with a disturbing situation worse than the surgical dilemma of the last decade or two. A debate for example, that CMF is categorically better than L-PAM or that CAMP (CTX, adriamycin, MTX, prednisone) is better than CMF could well rival the simple vs radical mastectomy polemic. Hopefully, since results are being obtained from carefully controlled studies in contrast to the method by which the surgical data were generated such controversies will be avoided.

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Chapter 14
The Role of Radiation Therapy in the Loco-Regional Treatment of Breast Cancer

R. CALLE

Radiation therapy plays an important role in the loco-regional treatment of breast cancer whether alone or following surgery. Preoperative irradiation has few advocates and will not be considered here.

Postoperative radiotherapy may be used following a radical, modified, or simple mastectomy, or after a tumorectomy. Its purpose is to sterilize small tumor foci and/or adenopathies that may remain following surgery.

The role of radiation therapy following radical mastectomy has been debated. Its effect has been mainly assessed by two therapeutic trials. The first, known as the Manchester trial (EASSON, 1967; PATERSON and RUSSEL, 1951) involved 1461 patients; the second, more recent, of the National Surgical Adjuvant Breast Project (FISHER, 1970), studied 1103 patients of which 428 have been followed for more than 5 years. The conclusions of these trials may be summarized as follows.

1. The incidence of local recurrences is decreased especially if irradiation of the thoracic wall is performed.
2. The 5-year survival rate remains unchanged.

Thus, radiation therapy following radical mastectomy may not be essential, as loco-regional recurrences can be treated at the time they occur. Furthermore, some authors such as STJERNSWARD (1974) claim that postoperative irradiation decreases the immunologic defense mechanism and hastens the appearance of metastases.

Radiation therapy has also been used systematically following simple mastectomy in a protocol established by McWHIRTER (1970). KAAE and JOHANSEN (1965) have compared extended mastectomy with dissection of the internal mammary and supraclavicular chains to simple mastectomy followed by irradiation: no significant difference in survival time was noted at 5 and 10 years for stage I patients. The incidence of local and/or regional recurrences was similar in both groups. In the trial of BRUCE (1971), radical mastectomy (204 cases) was compared to simple mastectomy followed by radiation therapy (191 cases) in patients with operable tumors of stages I, II, and III (Manchester classification). This trial showed a 10% difference in favor of radical mastectomy (76% vs. 66%); however, information concerning the repartition of patients according to stage in each of the treatment groups is lacking.

Radiation therapy can also be used following a tumorectomy.

Finally radiation therapy can be used alone not only in very advanced tumors but also in "operable" ones. These two conservative therapeutic modalities will be the object of this discussion. Based on the principles

and techniques described by BACLESSE et al (1960) and on the awareness that results remain essentially similar whatever the loco-regional approach and thus that other criteria should be considered such as the quality of cure we have applied these two forms of treatment since 1960 Between 1960 and 1969 we have treated 457 patients; their repartition according to the UICC TNM classification (1973) is shown in Table 1 Patients were under 75 years of age and presented with T1-T2-T3 tumors with or without clinically positive axillary nodes; tumors were equal to or less than 10 cm not fixed to the chest wall and without edema, skin infiltration or ulceration Bilateral tumors were excluded In this study N1b nodes are relatively rare because of the extreme severity of our criteria An adenopathy equal to or less than 1 5 cm without clear evidence of malignant characteristics was classified as N1a Since survival rates showed no difference between N0 and N1a both groups have been considered together Only lymph nodes with obvious clinical characteristics of malignancy were included in the N1b classification; in two-thirds of the cases cytologic confirmation was obtained

All cases were confirmed by histologic examination exceptionally by cytology Histology was obtained by tumorectomy or by drill biopsy

TUMORECTOMY AND IRRADIATION

This therapeutic method is being considered more and more frequently Among many publications those of BACLESSE (1960) MUSTAKALIO (1972), PAPILLON (1972) and PETERS (1968) must be cited

This therapeutic modality is extremely critized by numerous authors in view of the incidence of multicentric tumors that may reach 30-60% of the cases We agree with this notion; however, the evolution of these microscopic tumor foci is unknown and on the other hand irradiation is used as a means to sterilize them

From 1960 to 1969 we treated 101 patients by tumorectomy and irradiation with cobalt-60: 86 are alive at 5 years without evidence of disease progression and 79 have kept their breast (Table 2) Of 12 patients who had local and/or lymph node recurrences 7 were cured by surgery In 1 case a simple lymph-adenectomy was performed

Tumorectomy followed by irradiation should be used only within the frame of very strict rules concerning: (1) indications; (2) surgical technique; (3) irradiation technique

Table 1 TNM classification of 457 cases of mammary tumors treated by irradiation alone (356 cases) or by wedge resection and irradiation (101 cases)

	N0/N1a	N1b	Total
T0	0	8[a]	8
T1	56	0	56
T2	149	47	196
T3	99	98	197
Total	304	153	457

[a] Axillary tumors

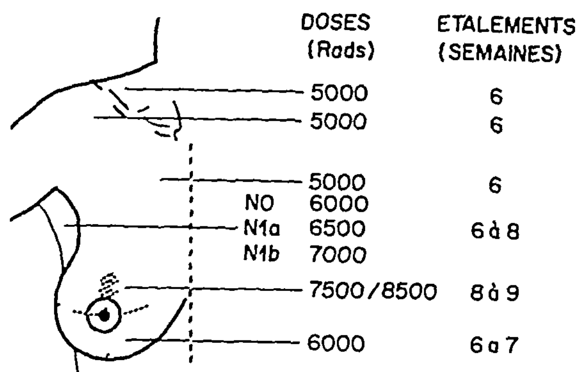


Fig. 1 Radiotherapy protocol (see text)

1. The tumor and lymph nodes remain stationary or do not regress appreciably; the irradiation is then discontinued and the patient is operated after a delay of 8-12 weeks.

2. The tumor and lymph nodes have distinctly decreased; irradiation therapy is then continued on more limited fields either by ^{60}Co or by electron beams, to a total dose of 8000-8500 rads to the primary tumor and 6500-7000 rads, exceptionally 7500 rads, to the lower axillary region. The diversity of these doses depends upon the volume and the radiosensitivity of the tumor and adenopathies.

3. If there are no clinically detectable adenopathies and if the breast tumor has not appreciably regressed, we tend to continue treatment only on the residual mammary tumor, preferably by electrons and up to maximum doses. Indeed, irradiation at high doses (8000-8500 rads) on a portion of the mammary gland does not prevent further surgery should such treatment be necessary. This is not the case if high doses are given to the axillary area.

At the end of the irradiation treatment, if the primary tumor and lymph nodes may completely regress, there are many cases where a residual nodule remains which may take several months to disappear. Consequently, when tumors are regressing, a minimal observation period of 4-5 months, somtimes of 9 months or more, is necessary before deciding in favor of a surgical intervention. Surgery is thus performed only in the absence of metastases, either for nonsterilization, for loco-regional recurrences, or in case of doubt.

Results

We have treated 356 cases according to this protocol with a follow-up greater than 5 years (Table 3). At 5 years 220 cases, or 60% are alive without relapse and one-half of these "cured" patients have kept their breast. We must insist on the fact that these 356 cases represent the most advanced potentially operable tumors; the totality of T1.NO tumors and the great majority of T2.NO tumors have been excluded as these patients were treated by tumorectomy followed by irradiation. The percentage of cures depends upon the TNM classification. It is of interest to note that whatever the classification, the proportion of "cured" patients who have kept their breast is approximately one-half.

Surgery for Radiotherapy Failures

Over half of the patients, namely 199, have required further surgery either for nonsterilization, for local or lymph node recurrences that sometimes appeared several years after the clinical and radiological

Table 3 Mammary tumors Irradiation as primary treatment (356 cases): Effect of T and N on results

	Number of cases	Survival at 5 years without recurrences	Conserved breasts
T2NO/NIa	108	84 (80%)	43
T2NIb	47	29 (62%)	12
T3NO/NIa	99	60 (60%)	26
T3NIb	98	43 (45%)	20
Axillary tumors	4	4	3
Total	356	220 (60%)	104

disappearance of the tumors and/or lymph nodes or exceptionally for painful retractile fibrosis of the mammary gland Two patients out of three or 126 are alive at 5 years without recurrences It would thus appear that these loco-regional recurrences do not alter the prognosis since we have obtained a 63% cure rate comparable to that obtained by primary surgical treatment

With regard to surgery it generally consitsted of a mastectomy with axillary dissection and depending on the location of the tumor with a dissection of the internal mammary chain In 13 cases a limited intervention was performed (tumorectomy or lymphadenectomy)

CRIITCAL EVALUATION OF CONSERVATIVE TREATMENTS

Tumorectomy plus irradiation and irradiation alone as primary treatment for breast cancer are justified only if the results are equivalent to those of standard surgical procedures if the postsurgical and postradiotherapeutic complications are acceptable and if a satisfactory cosmesis is obtained

Results

When all patients are considered the end results indicate that out of 457 cases 346 are alive at 5 years; of these 306 are alive at 5 years without recurrences (Table 4)

Table 4 Mammary tumors Conservative treatment (457 cases) Results

Results	Number of Cases
1 Survival at 5 years	346 (75%)
2 Survival at 5 years without recurrences	306 (67%)
Conserved breasts	183
a) By resection + irradiation	79
b) By irradiation + wedge resection	10
c) By irradiation alone	94

Among the patients apparently "cured", 183 or 2 out of 3 have kept their breast whether by tumorectomy followed by irradiation (79 cases), irradiation followed by tumorectomy (10 cases), or by irradiation therapy alone (94 cases). These 5-year results are comparable to those obtained by other forms of loco-regional treatments, particularly surgery (Table 5). It is also interesting to compare our results according to the presence or absence of axillary nodes (Table 6). They are strictly comparable, whether patients were submitted to a radical mastectomy (BUCALOSSI, 1971) or to a conservative treatment

Sequelae and Complications

Sequelae and complications are always difficult to evaluate as they often overlap; they have only been analyzed in patients treated from 1960 to 1966 inclusively. We have classified them into minimal (tolerable or transient), moderate (causing an undeniable but acceptable handicap), and severe (fibrosis of the pectoralis and of the supra- and infraclavicular area, brachial plexus palsy). These sequelae and complications have been summarized in detail in our prior publications (PILLERON et al., 1969; CALLE et al., 1973b).

We must insist upon the fact that it is the irradiation of the axillary region that may cause serious complications, as well as difficulties or impossibility of performing subsequent surgery. If very few serious accidents occurred among patients treated from 1960 to 1966, between 1969 and 1971, following a change in technique, we encountered several cases of radiation neuritis which led us to resume our former technique of irradiation.

Table 5. *Mammary tumors "operable" - results*

Authors	Number of cases	Treatment	Survival at 5 years (%)
BUCALOSSI et al , 1971	507	Extended mastectomy	67
URBAN, 1971	200	Extended mastectomy	72
URBAN, 1971	383	Radical mastectomy	72
WATSON, 1966	1149	Radical mastectomy	68
HAAGENSEN et al , 1969	482	Radical mastectomy	77
McWHIRTER, 1970	1091	*Mastectomy + irradiation*	62
KAAE, 1969	219	*Mastectomy + irradiation*	66
KAAE, 1969	206	Extended mastectomy	67
LACOUR, et al , 1969	1002	Miscellaneous	72

Table 6 Mammary tumors - 5-year survival according to the presence or absence of axillary lymph nodes

Authors	Treatment	Axillary node	Number of cases	Survival at 5 years (%)
BUCALOSSI et al (1971)	Extended mastectomy	N0NIa	327	72
		NIb	283	52
CALLE	Irradiation alone	N0NIa	304	72
		NIb	153	52

Esthetic Results

The esthetic results are very satisfactory and in some cases it is almost impossible to distinguish between the treated and nontreated breast Our best and worst esthetic results are shown in Figures 2 and 3 respectively

Disadvantages

The disadvantages result essentially from the irradiation technique the postradiotherapeutic surveillance and from the eventuality of having to decide for surgery



Fig 2 Right breast tumor with axillary metastases proven by cytology (T2N1b+) 10 years after irradiation with ^{60}Co



Fig 3 Left breast tumor with axillary metastases (T3N10) 2 1/2 years after irradiation with ^{60}Co

If the irradiation technique (CALLE et al., 1973a) is relatively easy after a wedge resection as irradiation at relatively moderate doses leads to few complications, this is not the case for primary irradiation, particularly for advanced tumors or for tumors associated with axillary nodes. The doses must be kept as low as possible without compromising the chances for cure. They must also insure the feasibility of subsequent surgery should irradiation fail to control the tumor.

In patients receiving primary irradiation the persistance of a residual tumor represents another difficulty; should surgery be performed and when? If the tumor remnant is well detected clinically and well visualized radiologically, the patient must be regularly and frequently examined as long as the tumor remnant regresses. However, if it persists or remains unchanged for several months, especially if the radiologic picture is typical of a neoplasm or if cytology reveals malignant cells, surgical intervention becomes imperative.

Unfortunately, as we have alluded to, this surveillance is sometimes very difficult due to the geographic location of the patients or to the presence of postradiation edema of the breast; some patients have been operated on unnecessarily because of doubts concerning the nature of the residual tumor: examination of the surgical specimen failed to reveal an active neoplasia.

We must also consider the incidence of distant metastases possible due to the unpaired immunologic defences that follows irradiation (STJERNSWARD, 1974). Among 457 cases we have noted a total of 89 metastases (Table 7); this does not appear to be an unusual number but further follow-up will be required. From 1970 to 1972, we carried out immunologic studies in 200 patients with breast carcinoma treated by irradiation (DABAN et al., 1975). These studies were performed immediately before and after irradiation therapy and at 3 months, 6 months and 1 year. We have observed a depression of the delayed hypersensitivity reactions to recall antigens and a decrease in the number of circula-

Table 7 Mammary tumor - conservative therapy (457 cases); patients with progressive disease or deceased - cause of failure

Cause of failure	Wedge resection and ^{60}Co	Irradiation	Total
Local recurrence, refused surgery	1	3	4
Acute local recurrence	0	7	7
Supraclavicular node recurrence	2	2	4
Chest wall recurrence	0	8	8
Local and metastatic recurrences	0	10	10
Metastases	9	80	89
Phenomenon associated with malignancy	1	0	1
Contralateral tumor	0	6	6[a]
Second primary	0	7	7
Intercurrent illness	2	4	6
Lost to follow-up	0	9	9
Total	15	136	151

[a]Plus 3 treated cases that remain alive

ting lymphocytes but the lymphoblastic transformation tests were not modified In general these tests returned to normal 4 months to over a year following irradiation However the significance of this immunologic depression is not known and with our current follow-up we were not able to correlate our immunologic monitoring with prognosis

CONCLUSION

The 5-year results of these conservative treatment methods are encouraging and similar to those obtained more with standard treatments in particular surgical ones Among the patients cured two out of three have kept their breast with a very satisfactory cosmetic result

Tumorectomy followed by irradiation appears to be an adequate therapeutic method providing the tumor is unifocal equal to or less than 3 cm nonevolutive and without axillary nodes

Irradiation as the primary modality for treating breast cancer is more complex and we do not encourage its general use; it requires a meticulous technique an important infrastructure from a diagnostic and therapeutic point of view and the close cooperation of the surgeon radiotherapist and patient One must not promise patients the conservation of their breast, but propose an attempt to that effect

SUMMARY

After having briefly considered the role of irradiation in the locoregional therapy of breast cancer the role of irradiation as a treatment modality aimed at conserving the breast is emphasized

A total of 457 patients were treated either by tumorectomy plus irradiation by ^{60}Co (101 cases) or by primary irradiation (356 cases) with a follow-up of over 5 years The results are encouraging and similar to those obtained by more standard treatments particularly by surgery Among the patients cured 2 out of 3 have kept their breast with a satisfactory esthetic quality

However the author insists on the difficulties presented by the irradiation technique on the necessity of a close cooperation between the surgeon and the radiotherapist and on the importance of a regular and frequent clinical mammographic and thermographic follow-up

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Chapter 15
The Role of Chemotherapy in the Treatment of Breast Cancer

P P CARBONE and P R BAND

In most occidental countries, breast cancer represents the commonest cancer in women; in the United States, it accounts for 28% of all malignancies. Mortality rate has shown little change over the past three decades (CUTLER and HEISE, 1969). Prognosis has not been influenced by various forms of surgery, whether extended, radical, or total mastectomy, nor by postoperative radiation therapy, performed as primary treatment for breast cancer (FISHER, 1970; FISHER et al., 1970). Therefore failure to cure the disease is not related to the removal and/or irradiation of the regional nodes. Over 50% of women having breast cancer will die of their disease within 10 years (FISHER et al., 1975a); particularly dire results are reported when the axillary nodes are involved with tumor (Table 1). Hence, in at least 50% of patients, when primary treatment is undertaken, the disease must be viewed as being systemic. In such cases, it would be logical to use a systemic form of therapy in addition to the loco-regional one. It is the purpose of this discussion to examine the role of chemotherapy, a systemic therapeutic modality, in the treatment of breast cancer.

THE ROLE OF CHEMOTHERAPY IN ADVANCED BREAST CANCER

As recently as 10 years ago, breast cancer was listed as a tumor poorly responsive to chemotherapy (KARNOFSKY, 1964). Traditionally, chemotherapy has been reserved for patients resistant to hormonal additive or ablative procedures. In these advanced cases, chemotherapy could only be palliative. Nevertheless, with the development of many new and active compounds, and through methodic clinical trials, knowledge has accumulated, concepts have evolved, and progress has been made. The effectiveness of single agents in the management of advanced breast cancer is well established (CARTER, 1974). Cumulative data indicate an

Table 1. Recurrence and survival rates after radical mastectomy for premenopausal and postmenopausal patients NSABP data (FISHER, 1970, FISHER et al., 1975a)

Axillary node status	Recurrence rate (%)		Survival rate (%)	
	At 5 years	At 10 years	At 5 years	At 10 years
Negative nodes	19	24	79	65
Positive nodes	67	76	45	25
1 - 3	51	65	62	38
≥ 4	81	86	30	13
All patients	42	50	63	46

objective response rate of 21-43% for the five most active and most frequently used drugs (Table 2) Despite a variety of single agents effective in patients with metastatic breast cancer rarely do complete responses occur, and rarely does the response last more than a few months Today the use of single agents has largely been supplanted by multiple drug combinations (BRODER and TORMEY 1974) We have learned from studies in acute leukemia and Hodgkin's disease that combination chemotherapy is superior to treatment with single drugs (FREI 1975) Two principles that underlie combination chemotherapy are: (1) the use of independently active agents with no overlapping toxicity in order to combine drugs at or near their maximal tolerated dose (2) the administration of intensive intermittent courses to ensure maximal tumor cell kill while enabling recovery from toxic or immunosuppressive effects (FREI 1975; HERSH and OPPENHEIM, 1967; HARRIS and STEWART 1972) The aim is not only to induce complete remissions in a majority of patients with advanced disease but also to develop effective and tolerable combinations that could safely be administered in conjunction with surgery and/or radiotherapy to patients with early breast cancer in order to delay recurrence and hopefully to cure the disease

In 1971, with the Eastern Cooperative Oncology Group (ECOG) we initiated in patients with metastatic breast cancer a clinical trial to answer this important question namely how the relative effectiveness and tolerance of combination versus single agent chemotherapy could usefully be translated to patients with early disease The schema of the study is shown in Figure 1 Two randomly allocated programs were examined (CANELLOS et al , 1974) L-phenylalanine mustard (L-PAM) given at the same dosage and schedule used in the National Surgical Adjuvant Breast Project (NSABP) adjuvant study (FISHER et al 1975b) was compared to a combination of cyclophosphamide methotrexate and fluorouracil (CMF) Table 3 illustrates the results for response rate and duration as well as for survival; the combination program was

Table 2 Single agent response rate in breast carcinoma (CARTER 1974)

Drug	Number evaluable	Response rate (%)
Adriamycin	102	43
Cyclophosphamide	529	34
Methotrexate	356	34
5-Fluorouracil	1236	26
Vincristine	226	21

Table 3 Eastern Cooperative Oncology Group study 0971 in advanced breast cancer Results

Therapeutic group	Response rate (%) Complete	Partial	Total	Median response duration (weeks)	Median survival all patients (weeks)
CMF[a]	15	38	53[c]	35	52
L-PAM[b]	5	15	20[c]	21	38

a 93 patients treated
b 91 patients treated
c p<0 01

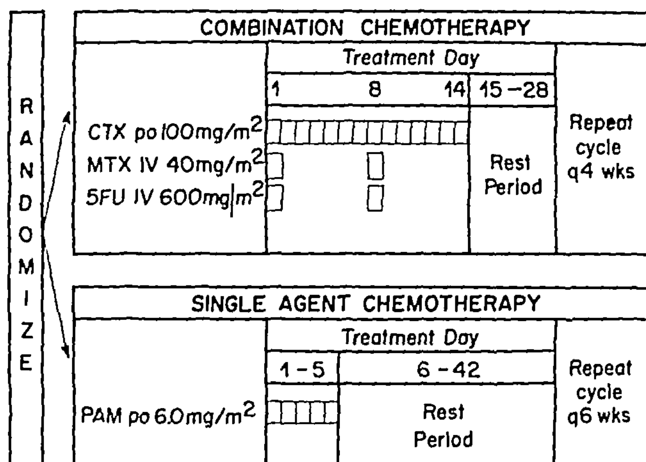


Fig. 1 Eastern Cooperative Oncology Group Study 0971 in advanced breast cancer Study design

superior in all aspects. Based on these data, the CMF combination has been evaluated in early disease (BONADONNA et al., 1975).

With the increasing use of multiple drug regimens in early disease, it has become important to recognize and to eliminate those drugs that carry the potential of producing debilitating toxicity on long-term administration without substantially adding to the effectiveness of the combination. AHMANN et al. (1974) reported that vincristine did not improve the response rate obtained with a three drug combination. Similarly, RAMIREZ et al. (1975) in a randomized trial comparing cyclophosphamide, methotrexate, vincristine, and fluorouracil with and without prednisone, found no difference in both groups. In a current ECOG study (unpublished observations), a comparison is being made between CMF with and without prednisone Preliminary data indicate no major difference. Thus, there is suggestive indication that vincristine and prednisone may not significantly add to the effectiveness of three or four drug combinations. Such is not the case, however, for adriamycin, very effective results have been obtained with this agent combined with cyclophosphamide (LLOYD et al., 1975), or with cyclophosphamide and fluorouracil (BULL et al., 1975).

DUCHATELLIER and ISRAEL (1971) predicted, on the basis of a mathematical model, the superiority of simultaneous combinations over the sequential administration of the individual drugs making these combinations. This theoretical demonstration was corroborated by two independent clinical trials. The Southeastern Oncology Group compared two five-drug combinations differing only in their scheduling, with the same five agents used sequentially on progression after each agent (SMALLEY et al., 1973). The Western Cooperative Group, in a similar design, also used a five-drug combination regimen versus the individual agents given sequentially (IRWIN, personal communication). In both studies, the simultaneous combination proved superior to the sequential administration of the single agents in terms of response rate, duration of response, and survival.

An urgent question needs to be answered, i.e., what is left for the patient when, after combination chemotherapy, relapse occurs? Two approaches have been undertaken to answer this important question. First, as discussed above, major efforts are being expanded to incorporate effective new drugs, such as adriamycin, into combinations. Second, several investigators are attempting to develop non mutually cross-resistant combinations. DE LENA et al. (1975) comparing CMF to adriamycin plus vincristine (AV) found the two regimens to be equal in effectiveness without being cross-resistant. The ECOG (unpublished ob-

servations) confirmed these results: patients were randomly allocated to receive either AV or CMF as primary treatment; those who failed on CMF were crossed over to the alternate therapy and vice versa Response rate was about 50% for both AV and CMF However of those patients who failed 30% were induced into remission when treated with the alternate regimen The identification of two independently active combinations offers the possibility to study the effect of sequential alternating drug combinations; furthermore the principles of induction and maintenance derived from studies in acute leukemias (HOLLAND 1971) can now fruitfully be evaluated in breast cancer Both approaches are currently being studied by the ECOG; we are also beginning to evaluate hormono- and immunotherapy in combination with chemotherapy The treatment Committee of the Breast Cancer Task Force is exploring the effect of combined hormono-chemotherapy for the treatment of first recurrences (CARBONE 1975)

As we have seen within a relatively short time advances in chemotherapy have improved the prognosis of patients with metastatic breast cancer; however the fact remains that most median survivals even with complete remissions last less than 2 years The potentially curative role of chemotherapy will likely reside in its application to patients with early disease in a combined modality approach

THE ROLE OF CHEMOTHERAPY IN EARLY BREAST CANCER

Kinetics studies in experimental animal tumor systems have indicated that the growth rate of solid tumors can generally be described by a Gompertz function (LAIRD 1964) and is characterized by a progressively longer volume doubling time as the tumor mass increases (SCHABEL 1969; SIMPSON-HERREN and LLOYD 1970) When large slow-growing tumors are uncrowded by transplantation their doubling time shortens their growth fraction increases (LASTER et al 1969; SCHABEL 1975) and the tumor cells become more vulnerable to various classes of chemotherapeutic drugs (SKIPPER 1971) These conditions are likely to prevail in micrometastases The relative failure of chemotherapy against advanced tumors may thus not only be due to drug resistance but possibly to kinetic resistance namely to the unfavorable kinetic parameters that exist in large slow-growing tumor Drug resistance implies the pessimistic view that the right durgs or the right combinations are not available The National Cancer Institute of the USA has screened thousands of compounds This year over 30 000 compound have been screened at a cost of millions of dollars If we do not have the right drug after all these years of screening then how many more years will we have to wait? On the other hand there is the possibility that we have the right drugs and that kinetic resistance rather than drug resistance is the major problem That such may be the case has been elegantly demonstrated by MAYO et al (1972) and SKIPPER (1974) at the Southern Research Institute in the experimental Lewis lung carcinoma This tumor metastasizes to the lungs within 7 days of subcutaneous transplantation into DBF_1 mice Surgical removal of the primary subcutaneous tumor on day 12 or treatment with cyclophosphamide on days 13 and 20 after implantation failed to cure any of the animals However surgical resection of the primary tumor on day 12 followed by cyclophosphamide on days 13 and 20 resulted in a 70% cure rate

The clinical implications based on these biological principles are obvious: systemic therapeutic regimens that are effective in advanced disease should be combined with local treatment surgery and/or radiation therapy at the time of initial therapy of early disease

Preliminary results from two recent clinical trials in breast cancer patients with positive axillary nodes will serve to illustrate how long-term adjuvant chemotherapy has significantly delayed recurrences. The NSABP in a double blind randomized trial, compared L-PAM given at a dose of 0.15 mg/kg daily for 5 consecutive days every 6 weeks for 2 years to a placebo. A significant delay in recurrence was initially observed for premenopausal women (FISHER et al., 1975b), and, after a longer follow-up, for postmenopausal patients as well (FISHER, personal communication). Toxicity was mild. While the effects on long-term results remain unknown, the prolongation of the disease-free period with a relatively mild treatment correlates well with the experimental data indicated above. In another study, Dr. Bonadonna, at the National Cancer Institute in Milan, Italy, working with the U.S. Breast Cancer Task Force, utilized the same basic study design as the NSABP but, instead of L-PAM, used the CMF regimen as outlined by the ECOG (vide supra) for a treatment period of 1 year. Preliminary data of this study have been reported by BONADONNA et al. (1975); updated results after 2 years of follow-up are reported here (Table 4). Of the 165 patients treated with surgery alone, there were 38 treatment failures. In the group that received adjuvant combination chemotherapy, there were 185 patients and only 5 treatment failures; the difference was highly significant. Most dramatic were the results in the 4 or more node group where treatment failure occured in 21 out of 50 patients in the surgery alone group and in only 3 of 61 patients in the CMF group; at 2 years an almost 50% recurrence rate was observed in the surgery alone group as opposed to a 95% disease-free survival for the chemotherapy treated group. These trials support the hypothesis that long-term systemic therapy can control micrometastases after primary surgery in high risk patients.

Through the task force which has sponsored Dr. Fisher's studies, a variety of adjuvant clinical trials are being done seeking to answer some important questions (CARBONE, 1975). How much therapy needs to be given and for how long? What is the role of hormone and/or immunotherapy?

SUMMARY

A wide variety of approaches are being applied to the therapy of breast cancer. Treatment begins with a biopsy followed by mastectomy to remove the primary tumor. The risk category must be determined and, at present, an axillary dissection appears to be required; in the future,

Table 4 Surgical adjuvant study (BONADONNA) Characteristics of patients with treatment failure

Characteristics	Evaluable patients				P value
	Control		CMF		
	No	%	No	%	
Total with recurrence	43/179	24.0	11/207	5 3	10^{-6}
Positive axillary nodes					
1 - 3	21/125	16 8	5/139	3 6	10^{-3}
≥ 4	22/54	40 7	6/68	8 8	10^{-4}
Premenopausal	20/82	24.3	5/95	5 2	10^{-3}
Postmenopausal	23/97	23 7	6/112	5 3	10^{-4}

tumor cell markers may replace the role of an axillary dissection in the determination of risk category (TORMEY et al 1975)

If the nodes are positive adjuvant chemotherapy and possibly immunotherapy should be considered A positive estrogen receptor assay suggests that patients may also benefit from endocrine treatments If it is negative the chances of responding to hormonotherapy are very limited except perhaps for anti-estrogens (McGUIRE et al 1975) Adjuvant therapy for patients with negative nodes is not recommended at this time; this view may have to be modified as the results of current adjuvant studies become available We have at hand the means to improve the cure rate of patients with breast cancer We are getting better diagnostic methods and find more patients with negative nodes We know more about the primary treatment and have systemic modalities that are effective in the adjuvant situation The immediate problem is to learn how to put these treatments together and this task has been undertaken by on-going clinical trials We are anticipating the results with optimism

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apter 16
e Role of Hormones in the Modern Treatment Advanced Breast Cancer

TAGNON

this chapter an attempt will be made to define the significance role of hormonal treatment of breast cancer This type of treat- t can no longer be used alone and it should be considered in the eral perspective of a resolute attempt to utilize all existing logical and pharmacologic information available for the improve- t of patient care This is not a complete review of the subject rather a chapter in the nomenclature and critical evaluation of hods of treatment now available to a task force with ambitious rapeutic aims Recent progress in the treatment of Hodgkin s dis- e leukemia osteogenic sarcoma and of other tumor types has pro- ed the encouragement for a renewed and concerted attack on breast cer a disease which affects 1 out of every 20 women and has a tality rate of probably over 75% which has practically remained hanged in the last 50 years

le the treatment of primary breast cancer has traditionally been ried out by surgical or roentgenologic methods the treatment of anced breast cancer up to a few years ago was largely the responsi- ity of endocrinologists especially the steroid endocrinologist inistration of hormones 30 years ago represented one of the first lly successful attempts at chemotherapy of a solid tumor It is not prising that much enthusiasm was generated by these early success- therapeutic trials and that intensive research continuing into contemporary period took place on the endocrine control of breast sue growth However in recent years the role of hormonal manipu- ion in the treatment of breast cancer has comparatively decreased importance The most recent clinical trials in our center as in ers either discard hormones altogether or at best assign hormones imited participation in multidrug regimens comprising up to five or e chemotherapeutic agents One question at present for the clinical estigator is to find out whether this participation of hormonal atment reduced as it is is necessary and should be maintained Al- ugh the more recently discovered and utilized antiestrogens appear have definite advantages over other types of hormonal treatments e think it not certain that they will be maintained as therapeutic nts in view of the rapid progress of nonhormonal chemotherapy Never- less it is of interest to review briefly the evolution of the fun- ental concepts underlying the use of hormones in breast cancer

hough the first regressions of breast cancer by the administration hormones were observed with the use of estrogens the systematic rch for active hormones was first carried out on androgenic com- nds (Council on Drugs 1961) No animal model was available for s search until rather recently Therefore it was based entirely on nical observation Around 1955-1956 the Cooperative Breast Cancer up described and applied a very precise methodology for the objec- e evaluation of results of treatment and we are still now using

practically the same criteria. A great number of androgenic compounds were tested on postmenopausal women and few, if any, were found to be superior to the reference compound testosterone propionate which gave from 15-20% remissions of an average duration of 6-9 months (Cooperative Breast Cancer Group, 1964; Groupe Europeen du Cancer du Sein, 1964). This modest achievement carried with it the heavy price of virilization with most of these compounds, affecting nonresponders as well as responders. Nonvirilizing androgen derivatives like Δ 1-testololactone were also developed, but alone or in combination with testosterone they did not improve the response (Groupe Europeen du Cancer du Sein, 1964).

While oophorectomy in premenopausal women produced a remission rate of approximately 30% (duration 9-15 months) the use of androgens in postmenopausal patients remained at a discouraging 20% rate of regression of limited (6-9 months) duration.

Other hormonal treatments were of superior effectiveness in specific instances. For example, high doses of estrogens for postmenopausal women were superior to androgens and less objectionable (Council on Drugs, American Medical Association, 1960). Estrogen effectiveness increased with the age of the patients and more specifically with postmenopausal age The apparent paradox of this effect of estrogens compared with oophorectomy is probably explained by the dosage; therapeutically used estrogens are administered at higher than physiological doses.

These relatively unsatisfactory results stimulated investigators to explore more radical methods of hormonal treatment such as bilateral adrenalectomy or hypophysectomy (FRACCHIA et al., 1971). The concept of hormone dependence, discussed elsewhere in this symposium, was at the basis of the introduction of these major ablative procedures. The number of responders increased slightly with these procedures but still remained well under 50%. Against this definite but somewhat mediocre therapeutic gain one had to weigh the operative risk and the inconveniences to the patient of a major endocrine imbalance, although this could be corrected by the administration of corticosteroids.

The inconvenience and the morbidity of these operations were obtained equally in responders and in nonresponders. Since nonresponders represented over 60% of all treated patients, these major ablative procedures imposed a heavy burden, without therapeutic benefit, in the majority of treated patients.

The justification for this therapeutic attitude was the lack of a better treatment (TAGNON, 1964) and the low operative mortality in experienced surgical hands.

However there was a need for the development of predictive methods for the selection of patients responding to hormonal treatment, and this need became more pressing as improved chemotherapy with its higher percentage of responders made it more and more difficult to recommend a major operation without a guarantee of at least an even chance of improvement.

Methods were proposed for such a prediction. BULBROOK et al. (1960) developed a "discriminant" based on a mathematical function derived from the measurement of urinary levels of 17-hydroxycorticosteroids and etiocholanolone. More recently, measurements of estrogen receptors in biopsy or surgical specimens of human breast cancer tissue have been introduced in the clinic for their predictive value. The extensive literature on this subject has been reviewed by McGUIRE et al

(1975) and LECLERCO et al (1975) Estrogen receptors as well as other steroid receptors represent a notable advance in the understanding of the biochemistry and biology of breast cancer which is bound to have an important influence on future therapeutic developments As far as the current situation is concerned the usefulness and clinical application of receptor measurements in breast cancer are in the process of being evaluated and concepts are rapidly changing It now seems that all patients regardless of the presence or absence of receptors should receive the same treatment The present evolution in the treatment of cancer of the breast is no longer a choice between hormonal or cytotoxic chemotherapy This is so because while hormonal research is making progress cytotoxic chemotherapy is also developing new concepts and possibilities Multidrug clinical trials have shown increased effectiveness of the association of drugs Also the introduction of new and powerful compounds such as CCNU and adriamycin as well as progress in preclinical and clinical pharmacology have resulted in a dramatic increase in the effectiveness of cytotoxic chemotherapy (STAQUET et al 1964) As a net result hormonal therapy became relegated to a secondary role while methods enabling some degree of selection of patient for hormonal treatment became available Furthermore with improvement of the technique for measuring estrogen receptors LECLERQ et al (1975) at the Institute Jules Bordet found receptors in an increasing percentage of breast cancer, albeit in variable amounts some tumors containing many receptor sites others containing very few Recently as many as 82% of all mammary cancers examined were found to be positive for estrogen receptors This marked predominance of receptor positive cancers appeared incompatible with a predictive usefulness of the measurement since fewer than 40% of the patients are known to respond to hormonal manipulation It is possible that rigorous quantitative assessment of receptor levels may result in the separation of a high level group always responding and of a low level group never responding to hormonal treatment with an intermediary group of unpredictable responses However, such a separation has not been demonstrated so far and it should be noted that concentrations of receptors in several hundred measurements represent a continuous spectrum and cannot be broken into two or more classes All one can say at this time is that receptor negative tumors may be negative only in a relative way and further refinement in the methods of detection might demonstrate that all breast tumors have some amount of receptors The same appears to be true for progesterone receptors which are now also measured in breast cancer (HORWITZ et al 1975)

In view of the rapid development of cytotoxic chemotherapy in the treatment of breast cancer estrogen receptor measurements although probably representing the most accurate evaluation of the degree of hormone dependence of a tumor may turn out to be of little practical value since treatment of advanced breast cancer no longer distinguishes between the hormonal and the cytotoxic approach However research on receptors remains very important because of the information provided on the essential hormonal factors at work in the genesis and maintenance of tumor growth

Interpretation of tumor growth or regression under hormonal treatment could be expressed in the following concepts or hypothesis based on the results of estrogen receptor measurements in humans for the clinician a tumor regresses when its volume decreases by a certain percentage (usually 50%); it is known that a 50% regression of a tumor mass may require a higher than 90% cell kill Under these conditions it may well be that only tumors containing an excess of receptors above a certain threshold will show a measurable decrease in size under hormonal therapy corresponding to a number of killed cells sufficient to produce a visible decrease in size These tumors are the so-called

hormone-dependent tumors of the clinician. In tumors having a lower concentration of receptors the number of cells killed under hormonal treatment could be insufficient to produce a measurable reduction in tumor size. These tumors would be called "hormone-independent". Actually, it is quite probable that all breast tumors are hormone-dependent for a fraction of their cells and hormone independent for the remaining fraction. This concept explains the extreme rarity of complete (even if temporary) regression of all lesions with hormonal treatment. Another implication of this concept is that certain hormonal treatment may be active despite the lack of measurable effects on the tumors.

It is well known that the hormone dependence of mammary cancer, when clinically recognizable, is always relative in the sense that regressions under steroid treatment or after ablative procedures are never complete and are of limited duration. The survey of estrogen receptors seems to reveal the biological basis for these clinical observations. Mammary cancer may tentatively be considered as essentially heterogeneous as fas as receptors are concerned. It contains a variable number of receptor negative cells and these are probably present in all cases and are not influenced by hormonal treatment. These cells probably constitute the emergence, after hormonal treatment, of recurrent tumors no longer sensitive to hormones. This concept accounts for the narrow limitations of hormone therapy and for the absolute necessity to add cytotoxic agents to any hormonal treatment Conversely there is every reason to add a hormonal agent of proven effectiveness to a cytotoxic regimen since a proprtion of cancer cells will be killed by this agent.

Therefore at the Institut Jules Bordet we now accept tentatively the concepts based on our estrogen receptor survey and consider that the practical application resulting from the ubiquity or near ubiquity of receptors in all breast cancers justifies the use of hormonal treatment of all patients concommitantly with chemotherapy. Our present therapeutic protocol for advanced breast cancer comprises a cyclic chemotherapy regimen associated with the continuous administration of a hormonal agent. Our chemotherapeutic regimen is analogous to that used at other institutions and consists of an association of agents which are known to be the most effective when given alone in advanced breast cancer The hormonal part of our therapeutic protocol is the antiestrogen tamoxifen. This agent, administered alone, gives a rate of objective remission of 35-40% with little or no toxicity and is therefore to be preferred to nafoxidin, another antiestrogen with similar activity but exhibiting a notable degree of toxicity especially on the skin. These antiestrogens appear to be at least equivalent therapeutically to high dosage of estrogen administration while being better tolerated and apparently free of the occasional tumor stimulating effect of estrogens (LECLERCO et al., 1975).

Whether the addition of an antiestrogen represents an improvement as compared to cytotoxic chemotherapy alone should be the subject of a controlled clinical trial in the future.

Other approaches to the treatment of advanced breast cancer by nonsteroid hormones, pituitary hormones, and neuroendocrine-active products remain, at present, experimental and have not yet proved useful in the clinic (STOLL, 1974).

CONCLUSION

Recent advances in chemotherapy with the introduction of new and potent drugs and the use of better combinations of drugs are based on the progress of clinical pharmacology and on the results of controlled clinical trials These developments have led to a re-evaluation of the treatment of advanced breast cancer and relegated hormonal treatment to the role of an adjuvant Endocrine treatment by administration of hormones as the only treatment may retain a few indications in elderly or very ill persons The major ablative procedures seem to be gradually replaced by the new medical treatments which give a larger percentage of remissions Ultimately the control of breast cancer will probably depend on the acceptance by the medical profession of early prolonged and energetic administration of active drugs to all patients undergoing surgical excision for breast cancer

Such treatment discussed elsewhere in this symposium has already produced spectacular results in reducing dramatically the rate of recurrences after mastectomy and should be generalized to all patients The type and intensity of surgical adjuvant therapy should be adapted to the stage of the tumor Early diagnosis by presently available methods with its small yield of new cases and high expenditure of money appears probably less important than support of research for the development of biochemical methods of diagnosis permitting detection before clinical manifestation Perseverance in the development and utilization of adjuvant therapy seems at present the most promising approach to prevent the appearance of metastases which when widespread and massive are much more difficult to eradicate or to treat Endocrine research and the exploration of the effect of hormones on breast cancer remain indispensable even if at present such studies may not directly influence treatment

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Chapter 17
The Role of Nonspecific Immunotherapy in the Treatment of Breast Cancer

L. ISRAEL

INTRODUCTION

Although many oncologists and immunologists at present consider the study and manipulation of specific immunity to be primordial we have devoted most of our efforts over the past 8 years to nonspecific immunostimulation in cancer patients for the following reasons

The first reason is a technical one and involves the present difficulty of assessing humoral and particularly cell-mediated specific immune reactions to solid tumors The second more important reason which we have stressed for some time is that the specific reaction which might be induced by a vaccine cannot really be expected to be effective in the immunodepressed host In the presence of nonspecific depression of all immune responses how could one expect the response to a specific stimulus to be enhanced? How could a patient who is incapable of developing an immune response to dinitrochlorobenzene respond to a tumor antigen particularly since the tumor which has grown unchecked has hitherto failed to elicit such a response? We felt that any attempt at immunotherapy should begin by restoring or enhancing general immunocompetence by nonspecific means before trying to manipulate specific immunity We postulated that such restoration if it proved possible would enable both enhancement of nonspecific antitumor mechanisms, such as activation of macrohpages and the development of specific reactions hitherto rendered impossible because of the inertia of the immune system in cancer patients

IMMUNE STATUS IN BREAST CANCER AND PROGNOSIS

Such immune inertia is prevalent in breast cancer patients and its extent correlates well with prognosis This is true in stage I cancers as was shown by ROBERTS and JONES-WILLIAMS (1974) using the skin test to varidase and by HOGE et al (1973) using the skin test to dinitrochlorobenzene and phytohemagglutinin- induced lymphocyte blastogenesis in vitro Similar findings had already been reported by WHITTAKER et al (1971) and by MITCHELL (1972) These investigations lend further support to the more general concept that prognosis in cancer patients is influenced by impaired immune responses

With regard to local and regional immune responses mention must be made of the work of BLOMGREN et al (1973) who showed that intratumoral lymphocytes did not respond to phytohemagglutinin (PHA) whereas circulating lymphocytes conserved their capacity to respond and of the investigation of FISHER et al (1972) who found that axillary node lympho-

cytes from breast cancer patients retain their capacity to respond to PHA.

The above data concern stages I and II breast cancer. When distant metastases are present there are usually more profound changes in immune status. In our personal studies we have found that over 80% of patients lose their ability to become sensitized to DNCB. This loss is usually accompanied by negative responses to skin tests with recall antigens and by a radical decrease in the PHA-induced blastogenic response of lymphocytes. Moreover, in the first investigation reported below, we found that the persistance of a positive response to PPD, despite widespread malignant disease, has always been associated with a better response to therapy and with longer survival. All the above data were strong motivation for putting nonspecific immunostimulation to the test in advanced breast cancer. Following an earlier study by ISRAEL and HALPERN (1972) which convinced us that Corynebacterium parvum was not only a potent immunostimulant, but that its subsutaneous administration to humans entailed no risk, we proceeded to use this agent in breast cancer.

I. Randomized Trial with Corynebacterium Parvum in Advanced Breast Cancer

I shall simply summarize this trial, the results of which were published at the Eleventh International Cancer Conference (ISRAEL, 1974a) (Table 1).

Eighty-two patients with lung, liver, or bone metastases, but not those with cerebral metastases or isolated metastases to the pleura or skin, were treated with a five-drug combination every 2 weeks (cyclophosphamide, methotrexate, fluorouracil, vinblastine, and rufocromomycin). After randomization, 43 of these patients were also given a subcutaneous injection of 4 mg of C. parvum (Merieux strain) once weekly. The results were as follows: the mean survival for patients on C. parvum (15 months) was 3 times as long as for controls (5 months), this difference was significant ($p<0.01$); response rates were the same in both groups. These patients were able to withstand higher doses of cytostatic drugs with a lower incidence of leukopenia and infection than control patients receiving chemotherapy alone.

Patients with a positive pretherapeutic skin test to PPD survived the longest, a finding which we had observed in previous studies.

II. Comparison of Two Nonrandomized Series

When the difference in the above trial became evident, we stopped randomizing patients and began treating all cases of advanced breast cancer with a three-drug combination (cyclophosphamide, methotrexate, and fluorouracil) given every 2 weeks in association with immunotherapy using i.m. or i.v. injections of C. parvum (Merieux) at a dose of 4 mg once weekly. However, owing to the fact that C. parvum is not commercially available and that it could only be administered at our institution, patients who, for geographic reasons, could not come frequently to our center were treated with the same regimen of chemotherapy by their family physician. These control patients were followed at our institution every 4-8 weeks

Fifty-two patients were treated with immunochemotherapy and 63 with chemotherapy alone. As this study was not controlled, the results must be viewed with some reservation; they remain, however, instructive.

Table 1 Randomized study of metastatic breast carcinoma Actuarial survival

Time from onset of therapy	Percent survivors Chemotherapy alone[a]	Chemotherapy plus C parvum[b]	Statistical significance at 12 months
3	78 4	100	
6	48 3	97 5	
12	41 1	85 1	p<0 001
18	31 9	54 5	
24	16	40 8	
30	0	32 7	
36	0	32 7	
42	0	0	

[a] 39 patients treated
[b] 43 patients treated

a T_3 and T_4 Primary Tumors
There were 11 patients in each group Survival was longer in the C parvum group throughout the observation period but never reached statistical significance (Table 2)

Table 2 Actuarial survival of T_3B-T_4 primaries

Interval (months)	Percent survivors Chemotherapy alone[a]	Chemotherapy plus C parvum[b]	Statistical significance
0 - 6	80	100	NS
7 - 12	80	100	NS
13 - 18	64	83 4	NS
19 - 24	64	83 4	NS
25 30	48	83 4	NS
31 - 36	48	83 4	NS

[a] 11 cases treated
[b] 11 cases treated

b Predominant Bone Metastases
There were 22 patients in the C parvum group and 16 in the control group No significant difference in survival was recorded The extent of bone involvement and the obligation to irradiate accessible lesions impaired bone marrow reserves thus making the administration of cytostatic drugs hazardous in both groups (Table 3)

c Predominant Lung Metastases
Although there were only 9 patients in the immunotherapy group and 16 in the control group we recorded a highly significant difference in survival in favor of the C parvum group at 6 12 and 18 months ($0\ 001<p<0\ 05$) The difference is no longer significant at 2 years but the actuarial probability of reaching 3 years is 40% as against 7% in the control group (Table 4)

Table 3. Nonrandomized study of breast carcinoma Actuarial survival. Diffuse bone metastases

Interval (months)	Percent survivors Chemotherapy alone[a]	Chemotherapy plus C parvum[b]	Statistical significance
0 - 3	100	95 3	NS
4 - 6	87 5	90 3	NS
7 - 9	68 7	85 3	NS
10 - 12	61 8	80 4	NS
13 - 15	51 62	63 2	NS
16 - 18	25 81	57 5	NS
19 - 21	25 81	25.5	NS
22 - 24	25.81	25 5	NS
25 - 27	12.90	25 5	NS
28 - 30	12 90	25 5	NS
31 - 33	12.90	25.5	NS

[a] 22 cases treated
[b] 22 cases treated

Table 4 Nonrandomized study of breast carcinoma Actuarial survival Pulmonary metastases

Interval (months)	Percent survivors Chemotherapy alone[a]	Chemotherapy plus C parvum[b]	Statistical significance
0 - 6	68 8	100	p<0 01
7 - 12	55.0	100	p<0 001
13 - 18	41 3	77 8	p<0 05
19 - 24	20 6	38 9	NS
25 - 30	6.8	38 9	NS
31 - 36	6 8	38.9	NS
37 - 48	6 8	38 9	NS

[a] 16 cases treated
[b] 9 cases treated.

d. Predominant Liver Metastases
Ten patients were treated with immunochemotherapy and 20 with chemotherapy alone. The difference in favor of C. parvum is significant at 12 and 15 months. At 24 months, 4 patients were surviving in the C. parvum group and none in the control group (Table 5).

e Analysis for All Sites
A significant difference in favor of immunotherapy was recorded at all time intervals from 6-42 months (Table 6).

Table 5 Nonrandomized study of breast carcinoma Actuarial survival Liver metastases

Interval (months)	Percent survivors Chemotherapy alone[a]	Chemotherapy plus C parvum[b]	Statistical significance
0 - 3	75	90	NS
4 - 6	53 6	70	NS
7 - 9	42 9	70	NS
10 - 12	21 45	70	p<0 01
13 - 15	14 15	50	p<0 05
16 - 18	14 15	40	NS
19 - 21	0	40	
22 - 24		40	
25 - 36		20	
37 - 48		20	

[a] 20 cases treated
[b] 10 cases treated

Table 6 Nonrandomized study of breast carcinoma Actuarial survival All metastatic sites

Interval (months)	Percent durvivors Chemotherapy alone[a]	Chemotherapy plus C parvum[b]	Statistical significance
0 - 6	70	89	p 0 01
7 - 12	50	86	p 0 01
13 - 18	27	59	p 0 01
19 - 24	20	38	p 0 06
25 - 30	13	34	p 0 05
31 - 36	2 7	27	p 0 02
37 - 42	2 7	27	p 0 02
43 - 48	2 7		

[a] 63 cases treated
[b] 52 cases treated

III Trial with Daily Intravenous C Parvum

We have recently reported our preliminary results with daily i v C parvum administered alone without chemotherapy in advanced cancer (ISRAEL et al 1975)

At the present time 46 patients including 6 cases of breast cancer have been treated for over 3 weeks Three objective partial responses have been recorded The duration of these responses could not be determined as whenever a partial response was documented patients were put on chemotherapy in the hope of inducing complete remissions

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